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The Journal of Laboratory and Clinical Medicine

Vol. 24 January, 1939 No. 4

CLINICAL AND EXPERIMENTAL

JUVENILE DIABETES MELLITUS

A Comparative Study of Standard Insulin, Crystalline Insulin, Protamine Insulin, and Hexamine Insulin

Henry M. Feinblatt, M.D., Brooklyn, N. Y.

CRYSTALLINE INSULIN

In 1926, Abel[1] prepared a crystalline insulin. This preparation of insulin, because of expense of production and technical difficulties, was unsuitable for use in our group of cases.

In 1934-35, Sahyun[2] produced a crystalline preparation of insulin with a low nitrogen content and an assay value of 25 to 25.5 units per mg. dry weight.

A group of 11 cases was selected from the diabetic clinic and admitted to the hospital for the study with crystalline insulin.[3] Six of the patients were between 10 and 17 years of age, 4 were between 20 and 25, and 1 was 50 years old.

The diets and insulin dosage were constant and identical with the patients' regular routine. The routine diets varied with relation to size, weight, and activity of the individual patient.

This study revealed that low blood sugars are sustained four hours longer with crystalline insulin than with standard insulin.

PROTAMINE INSULIN[4]

Out of a group of 400 active, ambulant, diabetic individuals treated with protamine insulin for over eighteen months, the first small group studied was selected for this report.

Diets and insulin dosage had been established. Blood sugar was determined at two-hour intervals, and comparative graphs of the blood sugar levels on regular and protamine insulin were made throughout the experimental period.

Received for publication, March 23, 1938.

The diets were prescribed to conform with the habits of the patient and the family and included a late meal at bedtime. The low ratio was 3 carbohydrates to 1 of protein and 1 of fat; the high ratio 5 carbohydrates to 1 of protein and 1 of fat. The entire group showed a gain in weight, and in some the diet had to be restricted to prevent obesity.

The unit value of protamine insulin was found to be greater and more lasting than the unit value of standard insulin. Our dosage was calculated upon the basis of 80 per cent of the dose of standard insulin.

We were pleased by the absence of ketonuria and the decreased incidence of lipemia in this group under protamine insulin therapy, especially as the presence of acetone breath and acetonuria was almost a constant finding in these diabetics controlled with standard insulin.

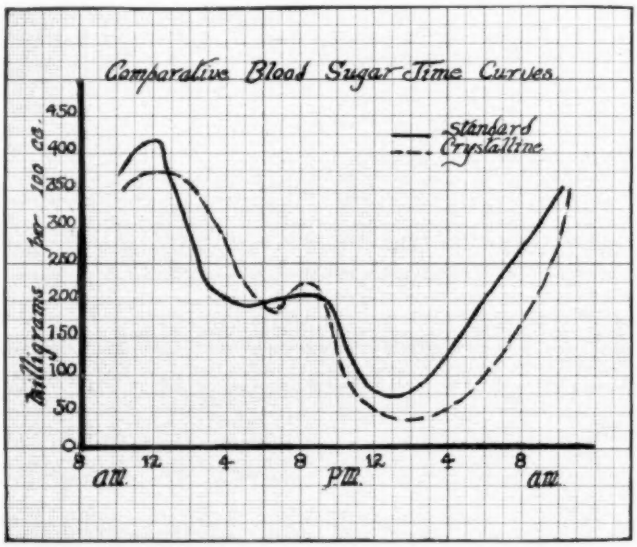

Fig. 1.—Composite graph of 11 cases showing blood sugar levels at two-hour intervals under administration of standard insulin and crystalline insulin.

Obviously, this insulin precipitate is remarkably effective in stimulating the completion of fat metabolism and thus eliminating ketonuria from diabetes.

Our juvenile diabetics frequently showed blood sugar levels below 60 mg., and were entirely free of symptoms at such levels. This level was, therefore, taken as a routine safe and comfortable margin for those under protamine insulin therapy.

A modification of this level was considered for those patients of middle age or over, with or without cardiac disease or hypertension, because this type commonly reacted at sugar levels in the neighborhood of 70 mg. For these cases a low level of 100 mg. has proved most satisfactory.

Blood sugars were examined at two-hour intervals throughout the twenty-four hours, and an average was determined for each case and for the group.

The curve of periodic variations in a group of 30 cases of juvenile diabetes revealed a continuous sustained drop which reached its lowest level at midnight.

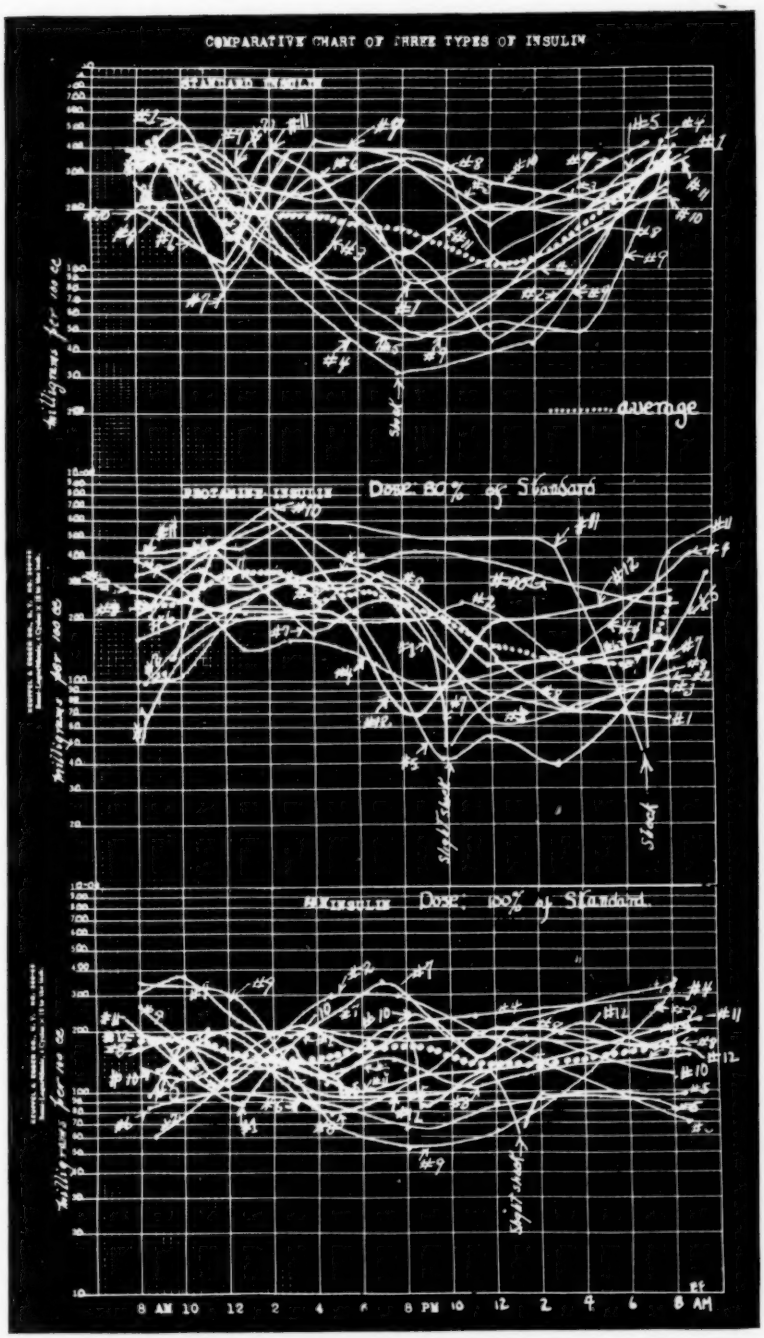
COMPARATIVE CHART OF THREE TYPES OF INSULIN
STANDARD INSULIN
average
PROTAMINE INSULIN Dose: 80% of Standard
HEXINSULIN Dose: 100% of Standard
8 AM 10 12 2 4 6 8 PM 10 12 2 4 6 8 AM

Fig. 2.—Eleven cases treated successively with standard protamine and hexamine insulin. Same number refers to same patient in each graph.

The average blood sugar level was 177 mg. Claude Bernard's threshold value of 170 mg. was decided upon as the average optimum diabetic threshold level.

During the first twenty-four-hour period after injection of protamine insulin, a drop in blood sugar began during the fifth hour and continued to the end of the twenty-four hours. During the second twenty-four-hour period, the sugar curve showed a continuous rise after the morning injection which reached a plateau in five hours and was followed by a drop which reached its lowest level twenty-four hours after injection.

The average curve on protamine insulin therapy, as shown on the graph by the dotted line, demonstrates a sinelike form. This makes it possible for the application of a formula which would show a direct relationship between the high and low peaks and the daily average levels.

Protamine insulin adjustment was established at the end of the third day. The unit value of protamine insulin was found to be 20 per cent greater than the unit value of regular insulin. In the group studied, the intercipient use of standard insulin conflicted with the efficiency of protamine insulin and was eliminated.

The introduction of the protamine preparation of insulin was a tremendous contribution to the treatment of diabetes. The practicality of a single daily injection encouraged both the patient and the physician to favor insulin therapy.

There has been, however, some hesitancy on the part of the medical profession to fully utilize this new therapeutic factor because of the following reasons:

1. Fear of allergic manifestations. Such reactions have occurred in our group and are decidedly more common with protamine insulin than with standard insulin.

2. Delayed response. On the basis of the average, there is an immediate response in a lowering of the blood sugar with standard insulin. With protamine insulin, the response is not evident until the sixth hour. Because of this delayed response, the rise in blood sugar caused by breakfast is not checked by protamine insulin. Most investigators found it necessary to use intercipient doses of standard insulin to check this early rise. This often resulted in a summation effect expressed in insulin shock.

3. Insulin shock. Attempts to lower the average blood sugar levels by increasing the dose of protamine insulin (because of the sinelike character of the blood sugar curve) also lowers the low points and results in hypoglycemia.

4. Character of the precipitate. The precipitate, on standing, packs, and it is only with difficulty that it can be shaken into a homogeneous mixture. Strengths higher than U-80 clog the 27 gauge needle, and the larger needles are objectionable.

5. Local reactions. Annoying local reactions occur at site of injection of more than 1 c.c. protamine insulin. They are in the form of hard, firm, inelastic nodules, which persist two or three days and are gradually absorbed without discoloration.

6. Character of shock. Shocks caused by protamine insulin, as compared with those produced by standard insulin, last longer and do not respond as quickly to the ingestion of carbohydrates.

HEXAMINE INSULIN[5]

In the course of our investigations, one of my co-workers, Dr. Warburton, developed a compound of insulin and hexamethylene tetramine using 0.25 grains of hexamine to 1000 units of insulin.

The particles of the precipitate were very fine and could readily pass through the 27 gauge needle in strengths as high as U-1000.

We then found that this precipitate could be dissolved on either the acid or alkaline side and that it would reprecipitate at the pH of the body tissues.

This preparation could be buffered and was nonirritating. The alkaline-buffered solution was as stable as protamine insulin and, when cleared, could be used in any concentration.

Fourteen of the most severe cases of juvenile diabetes were selected for the study of hexamine insulin. This group included those patients treated with standard, crystalline, and protamine insulin.

The dosage of insulin and diet which we have found necessary to maintain these patients in a satisfactory mental and physical state was used as a criterion for this study. In each case the hexamine insulin was administered in a single daily dose. Blood sugar determinations were made at two- and four-hour intervals throughout the twenty-four hours.

An average of 12 cases on regular insulin showed a drop of 200 mg. in blood sugar four hours after the first injection and another drop of 200 mg. six hours after the second injection.

Protamine insulin resulted in a low morning peak of 120 mg. at 6:00 A.M., and blood sugar levels of 230 mg. and 250 mg. at 8:00 A.M. The first eight hours after injection of protamine insulin were characterized by high sustained levels, the average being 350 mg.

With hexamine insulin the average morning blood sugar level was 180 mg. In general, with this insulin the average curve fluctuates between 140 and 170 mg. throughout the entire day.

The absence of ketonuria was as striking with hexamine insulin as with protamine insulin. This insulin also has a specific effect in decreasing polyuria in those patients who complained of it both with standard and with protamine insulin. Shocks caused by hexamine insulin respond immediately to the ingestion of small amounts of carbohydrates.

A single dose of hexamine insulin may be substituted for two to four divided doses of standard insulin. One dose of hexamine insulin produces a blood sugar curve similar to the one produced by four divided doses of standard insulin, but with decided constriction in the range fluctuations of blood sugar.

All the hexamine insulin used was prepared by Edgar A. Ferguson.
The author is indebted to Dr. Alpert for his clinical and laboratory assistance.

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ATMOSPHERIC POLLEN OF NASHVILLE, TENN.*

EVANGELINE BOWIE, M.D., NASHVILLE, TENN.

NASHVILLE is located in the middle Tennessee Basin. A record of the pollen content of the air was obtained from an elevated station on the campus of Peabody College, which is located in the western residential section of the city. This elevation is in the path of winds bringing in pollen from the wooded hills around the city.

The technique employed for catching atmospheric pollen, and the method of counting are described by Wodehouse.[1, 2] Melted fuchsin glycerin jelly was placed on slides to cover an area equal to a No. 0 cover glass. Exposures were made in a horizontal position on the roof of a five-story dormitory. Slides removed were warmed, and in order to prevent flattening of the pollen grains, it was often necessary to add an additional drop of melted jelly before applying the cover glass.

The relative abundance of fall elm pollen is shown in the accompanying graph for the fall of 1936 and 1937. The source of this pollen is a fall flowering elm, *Ulmus serotina* Sargent, which is native to middle Tennessee and Kentucky. Gattinger[3] states that it was discovered in 1878 near the grounds of Vanderbilt University. The increase in amount of fall elm pollen and the decrease of ragweed pollen during 1937 as compared with that of 1936 may be explained by a difference in rainfall. During the growing season of 1937, the rainfall was sufficient to keep the trees in heavy foliage, and the effect of shade may have been a factor in the decrease of ragweed.

Black and Durham[4] state that fall blooming elms of the South may cause some perplexing problems during the ragweed season. Their chart of pollen counts in Dallas, Texas, shows fall elm pollen from cedar elm, *Ulmus crassifolia* Nutt., reaching a peak during the second week of September, 1929. Chinese elm, *Ulmus parvifolia* Jacq., which flowers in the summer or fall, has been introduced into the United States, and may become of some importance in the future as a source of elm pollen.

The first grains of giant ragweed *(Ambrosia trifida)* are caught around August 15. Later the grains of dwarf ragweed *(A. elatior)* appear.

Artemisia pollen is one of the most abundant varieties of fall pollens. *Artemisia annua* is found in the city and is commonly called Sweet Annie, sweet wormwood, or annual wormwood.

Chenopod includes the pollen of lamb's quarters *(Chenopodium album)*, Mexican tea *(C. ambrosioides)*, Jerusalem oak *(C. botrys)*; pigweeds *(Amaranthus hybridus* and *A. spinosus)*; and burning bush *(Kochia scoparia* Schrad.).

*Read before the Botany Section at the Forty-First Meeting of the Tennessee Academy of Science, Nashville, Tenn., Nov. 26, 1937.

Received for publication, April 4, 1938.

Grass pollen is found in small amounts from spring to frost. Blue grass *(Poa pratensis)* and orchard grass *(Dactylis glomerata)* flower in April and May. Rye grasses *(Lolium perenne* and *L. multiflorum)* are planted for a winter cover, and bloom in May and June. Bermuda and Johnson grass bloom during the summer and fall. Other species of grass which are contributory to hay fever occur in this region.

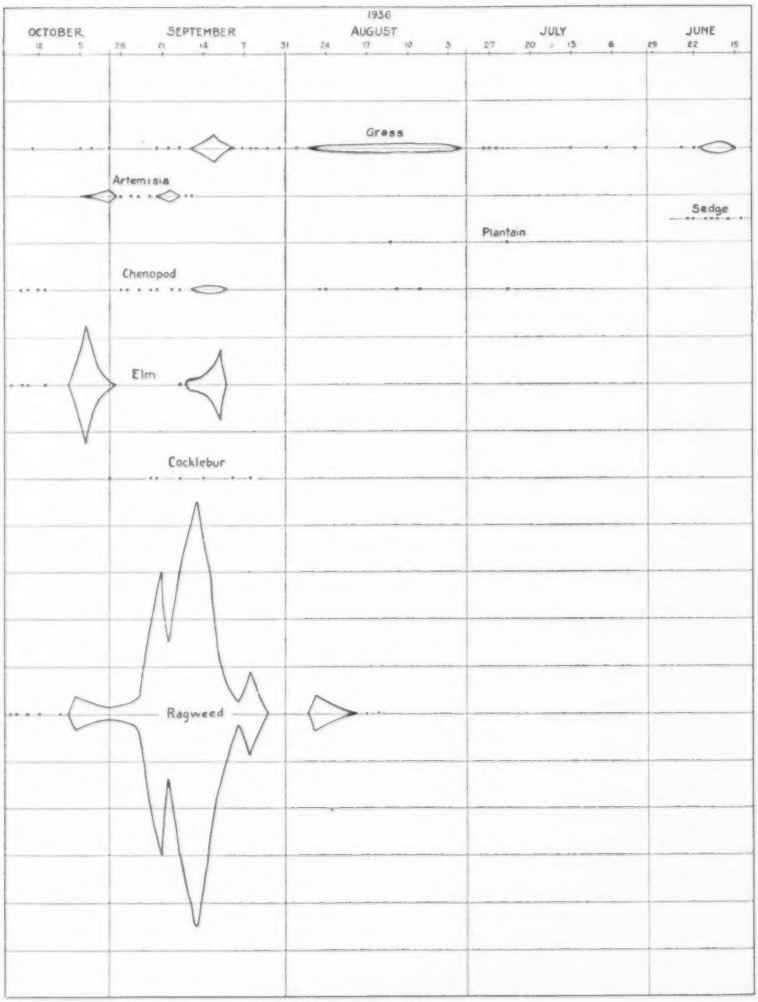

Chart 1.—Record of the atmospheric pollen of Nashville, Tenn., during the summer and fall of 1936 and the year of 1937. The distance between the horizontal lines represents 16 grains of pollen caught on an area of 3.5 sq. cm. during twenty-four hours (Continued on pages 344 and 345).

During 1937 the percentage of tree pollens was larger than that of grass and weed pollens. Spring rains and rapid changes in temperature account for sharp peaks and depressions of tree pollens.

Elm pollen is one of the earliest spring pollens. Some anthers may be found shedding pollen during warm days in January. Winged elm *(Ulmus alata* Michx.), American elm *(U. americana),* and slippery elm *(U. fulva*

Michx.) are native spring blooming elms. Dwarf elm *(U. pumila)*, commonly called Chinese elm, was introduced about ten years ago, and numbers of these trees are now blooming in the spring.

The total maple count is low. Acquarone and Gay[5] found, by exposure of slides near maple trees, that maple pollen was not carried more than a few rods by ordinary breezes.

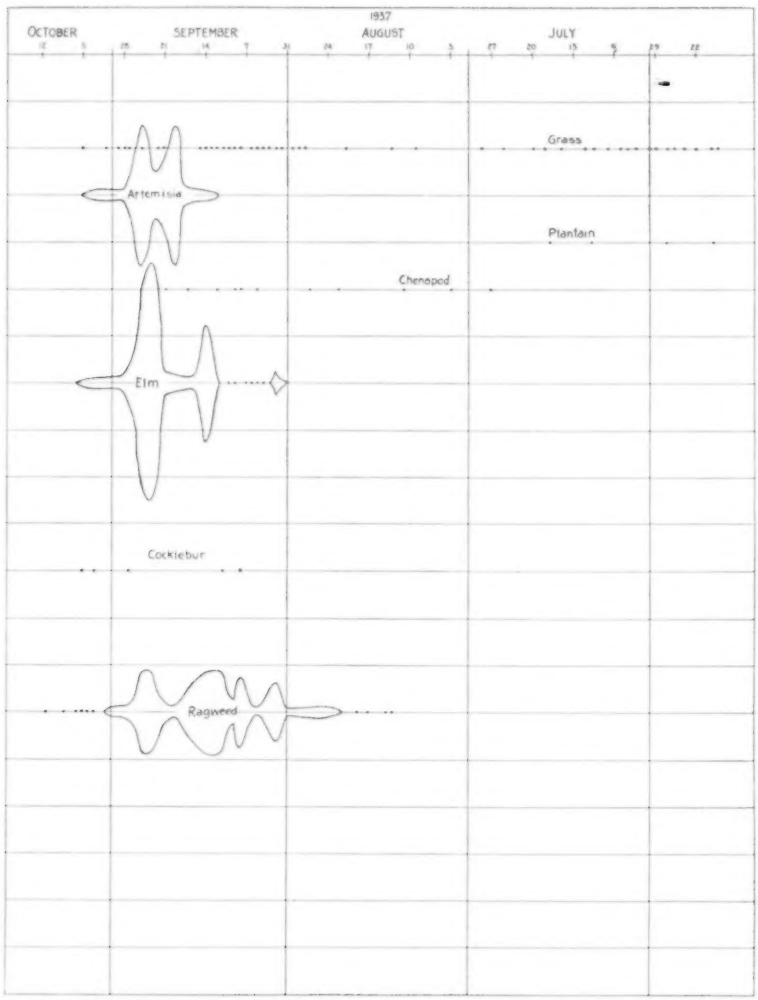

Chart 1.—Cont'd

Silver leaved poplar *(Populus alba)* blooms early in the spring, and is followed by cottonwood *(Populus deltoides* Marsh.).

Juniper: Red cedar *(Juniperus virginiana)* grows in the cedar glades of middle Tennessee.

Hornbeam: The pollen of the hornbeam, *Carpinus betula*, and the hop hornbeam, *Ostrya virginiana* Willd., is included under this title.

Practically all the different species of oak are represented, which accounts for the long period during which oak pollen is found.

Birch trees occur very sparingly and the pollen is negligible.

A large quantity of beech pollen is blown into the city from the surrounding woods.

Hackberry trees are very abundant in Nashville. Pollen of the two species, *Celtis occidentalis* and *C. mississippiensis* Bosc., was not differentiated.

Maclura: Osage orange *(M. pomifera* Schneid.) sheds a large amount of pollen during May.

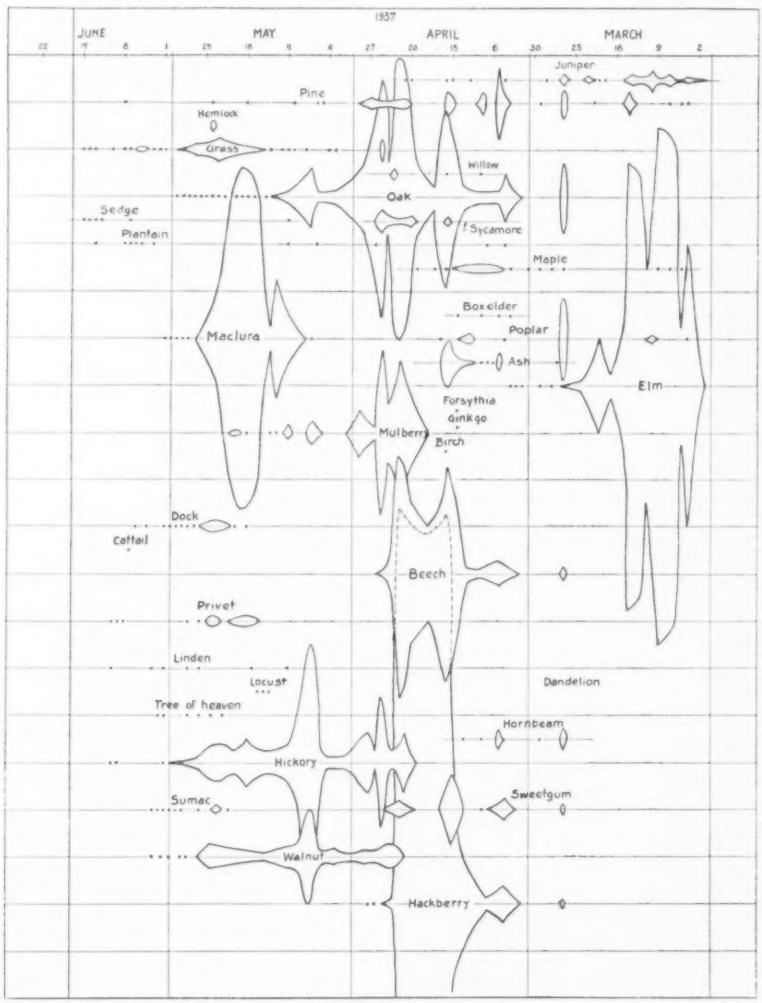

Chart 1.—Cont'd

Mulberry: Under this head are included white mulberry *(Morus alba)*, red mulberry *(M. rubra)*, and paper mulberry *(Broussonetia papyrifera)*. The pollen of paper mulberry predominates. Bernton[6] has shown that osage orange, white and red mulberry, and paper mulberry have a generic relationship in causing hay fever.

Walnut and hickory pollens closely parallel each other in time of appearance. Black walnut *(Juglans nigra)*, white walnut *(J. cinerea)*; shagbark

hickory *(Carya ovata* Mill.), mockernut *(C. alba)*, and pignut *(C. glabra* Mill.) are found in the city and surrounding woods.

Sycamore: *Platanus occidentalis* and *P. orientalis* are along the driveways of the campus.

Privet *(Ligustrum vulgare)* is used on the campus for hedges, and allowed to bloom profusely.

SUMMARY

A study of the atmospheric pollen of Nashville, Tenn., during the summer and fall of 1936 and the year of 1937, is presented in the form of a chart and briefly discussed.

Attention is called to the presence of fall elm pollen in order that its importance in allergic conditions may be considered.

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THE EFFECT OF COPPER AND IRON UPON THE SECONDARY ANEMIA OF THERAPEUTIC MALARIA IN GENERAL PARESIS*

Purcell G. Schube, M.D., Boston, Mass., and
Blake D. Prescott, M.D., Hartford, Conn.

THE value of copper as a supplement to iron in the regeneration of hemoglobin was demonstrated first on anemic rats in 1928 by Hart, Steenbock, Waddell, and Elvehjem.[1] They showed rather conclusively that soluble inorganic iron salts in the presence of copper could be used to stimulate hemoglobin formation. Since the publication of this work, a number of other individuals have studied the effect of copper and other metals in conjunction with iron on hemoglobin production in anemic animals and in man. Most of these studies have verified the conclusions of Hart so that today, although it has been demonstrated that copper is not the only element which, when given with iron, stimulates hematopoiesis, practically everyone is in agreement that copper can be a most active catalyst in the stimulation of hemoglobin synthesis.

In the course of our work on the treatment of general paresis with malaria, we have been frequently annoyed and worried by the secondary anemias which almost invariably resulted. At times these anemias were exceedingly alarming, not only in their intensity, but also in the persistency with which

*From the Boston State Hospital, and the Neuro-Psychiatric Institute and Hospital of the Hartford Retreat.
Received for publication, April 14, 1938.

they remained after the termination of the malaria, and irrespective of the institution of ordinary iron or liver therapy. The combination of copper with iron was instituted in some of these cases, and the results being sufficiently promising, it was decided to study the problem carefully. It is the purpose of this paper to present the results of this study.

METHOD

In each instance the procedure was the same. The hemoglobin and red cell counts were determined weekly before, and throughout the administration of the copper and iron. The drugs* were always administered orally in gelatinous capsules, a capsule being given three times daily. Each capsule contained 32 mg. of iron and 1.0 mg. of copper. The red blood cells were counted by the usual pipette and counting chamber method. The hemoglobin was determined chemically.

The controls were of three types: (1) a group of patients with anemias of unknown origin; they were given the copper and iron and the rate studied at which the red blood cells and the hemoglobin returned to normal; (2) a group of patients with general paresis; they were given therapeutic malaria but no copper and iron, and the progression and regression of the resulting anemia carefully evaluated; (3) a group of general paretics who were given malaria therapy and at the same time 32 mg. of ferrous iron until their anemia had disappeared.

RESULTS

Eleven adult individuals with varying grades of secondary anemia were given the copper and iron capsules as described. The effect of this treatment upon the anemia of the group is shown in Chart 1.

Fourteen adult general paretics were given malaria as a therapeutic measure. The effect of the malaria on red cell counts and hemoglobin of the group is shown in Chart 1.

Thirty-one adult general paretics were inoculated with therapeutic malaria, and the iron treatment was started at once. The effect of the iron on the anemia of the group is shown in Chart 1.

Thirty-seven adult general paretics were inoculated with therapeutic malaria, and the copper and iron treatment started at once. The effect of the combination of these two drugs on the resulting anemia of the group is shown in Chart 1.

A survey of this material brings out the following facts:

Group 1. Secondary Anemia of Unknown Origin.—In this group of cases, 8 responded to the copper and iron with increases in red blood cell counts of from 560,000 to 1,070,000 over a period of from two to fourteen weeks. Eight responded with increases in hemoglobin of from 10 to 20 per cent over a period of two to fourteen weeks. Usually the increases in the hemoglobin and red blood cells accompanied each other, although the increases were not all proportionate. The copper and iron in the quantity used, therefore, does

*The copper and iron preparation used was Copperin "A", manufactured by Myron L. Walker, Inc.

increase very definitely the percentage of hemoglobin and number of red blood cells in secondary anemia. It is felt that the ability of these two chemicals to decrease a secondary anemia being established for this study, any differences observed in groups 2, 3, and 4, are probably due to copper and iron.

Group 2. Induced Secondary Anemia in Malaria-Treated General Paretics but Untreated With Copper and Iron.—In this group the hemoglobin reached its lowest point in four to eight weeks after the first temperature elevation. In one case the lowest hemoglobin was obtained one week before the malaria was terminated; in 2 it was reached in the same week the malaria was terminated; and in 11 cases the lowest hemoglobin was reached one to five weeks after the malaria had been terminated. The time required for the hemoglobin to reach a concentration of 80 per cent from the termination of the malaria was four to eleven weeks. The lowest hemoglobin percentage in the cases ranged from 36 to 58 per cent. The red blood cell count reached its lowest point in three to ten weeks after the first temperature elevation. In one case the lowest red blood cell count was obtained one week before the malaria was terminated; in 3 it was obtained on the same week the malaria was terminated; and in 10 cases the lowest red blood cell count was reached one to six weeks after the malaria was terminated. The time required for the red blood cells to reach their highest concentration from their lowest concentration was four to eight weeks. The time required for the red blood cells to reach their greatest concentration from the termination of the malaria was four to eleven weeks. The lowest red blood cell concentration in the group was 1.98 to 3.51 millions. The changes in the red blood cell concentration roughly paralleled the changes in the hemoglobin percentage.

Group 3. Induced Secondary Anemia of Malaria-Inoculated General Paretics Treated With Iron.—In this group the hemoglobin reached its lowest point in three to eight weeks after the first temperature elevation. In 2 cases the lowest hemoglobin was obtained one week before the malaria was terminated; in 5 it was reached on the same week the malaria was ended; and in 24 cases the lowest hemoglobin was reached one to three weeks after the malaria was terminated. The time required for the hemoglobin to reach a concentration of 80 per cent from its lowest concentration was one to nine weeks. The time required for the hemoglobin to reach 80 per cent from the termination of the malaria was one to ten weeks. The lowest hemoglobin percentages in the cases ranged from 37 to 68 per cent. The red blood cell count reached its lowest point in three to eight weeks after the first temperature elevation. In 4 cases the lowest red blood cell count was obtained one week before the malaria was terminated; in 9 it was obtained in the same week the malaria was ended; and in 18 cases the lowest red blood cell count was reached one to four weeks after the malaria was terminated. The time required for the red blood cell count to reach its highest concentration from its lowest concentration was one to nine weeks. The time required for the red blood cell count to reach its greatest concentration from the termination of the malaria was one to ten weeks. The

lowest red blood cell concentration in the group was 2 to 3 million. The changes in the red blood cell concentration roughly paralleled the changes in hemoglobin percentage.

Group 4. Induced Secondary Anemia of Malaria-Inoculated General Paretics Treated With Copper and Iron.—In this group the hemoglobin reached its lowest point in one to five weeks after the first temperature elevation. In 4 cases the lowest hemoglobin was obtained one week before the malaria was terminated, and in 33 it was reached one to three weeks after the malaria was terminated. The time required for the hemoglobin to reach a concentration of 80 per cent from the lowest concentration was 0 to five weeks. The time required for the hemoglobin to reach 80 per cent from the termination of the malaria was one to three weeks. The lowest hemoglobin percentages in the cases ranged from 47 to 77 per cent. The red blood cell count reached its lowest point in one to four weeks after the first temperature elevation. In 13 cases the lowest red blood cell count was obtained one week before the malaria was terminated; in 4 it was obtained in the same week the malaria was ended; and in 20 cases the lowest red blood cell count was reached one to two weeks after the malaria was terminated. The time required for the red blood cell count to reach its highest concentration from its lowest concentration was one to five weeks. The time required for the red blood cell count to reach its greatest concentration from the termination of the malaria was one to five weeks. The lowest red blood cell concentration in the group was 2.5 to 4 million. The changes in the red blood cell concentration roughly paralleled the changes in hemoglobin percentage. There was apparently no relationship between the number of chills induced by the malaria and the changes in the hemoglobin and red blood cell counts.

Chart 1 illustrates the average trend of the hemoglobin values in groups 1, 2, 3, and 4. The red cell curve so closely paralleled the hemoglobin curve that this latter is not presented.

Chart 2 illustrates graphically the essential facts presented in groups 1, 2, 3, and 4.

DISCUSSION

A general survey of this study would tend to establish in our cases the following facts: (1) The administration of copper and iron does stimulate hemoglobin and red blood cell formation. This is well illustrated in the cases of group 1. In these cases the combination increased the hemoglobin and red blood cells quite consistently in most instances. (2) In malaria-treated general paretics (group 2) the hemoglobin and red blood cells were both depressed, and increased only after the termination of the malaria. This return to normal was a slow process, often requiring many weeks. (3) In malaria-treated general paretics (group 3) when ferrous iron was administered regularly from the time of the malarial inoculation to the return of the blood picture to normal, the average curves of depression and recuperation of the blood picture were approximately the same as those in group 2.

When, however, the lowest hemoglobin and red blood cell values and their distances from the first temperature elevation were studied, it was found that there was a definite shift of some of these low values away from the first temperature elevations. And when the distances from the lowest hemoglobin and red blood cell counts to the last (or first normal) hemoglobin and red blood cell counts were studied, it was observed that the distance (time) of hemoglobin and red cell recovery was lessened. The secondary anemias of group 3, moreover, were not as profound as those of group 2. (4) In malaria-treated general paretics (group 4) when copper and iron were regularly admin-

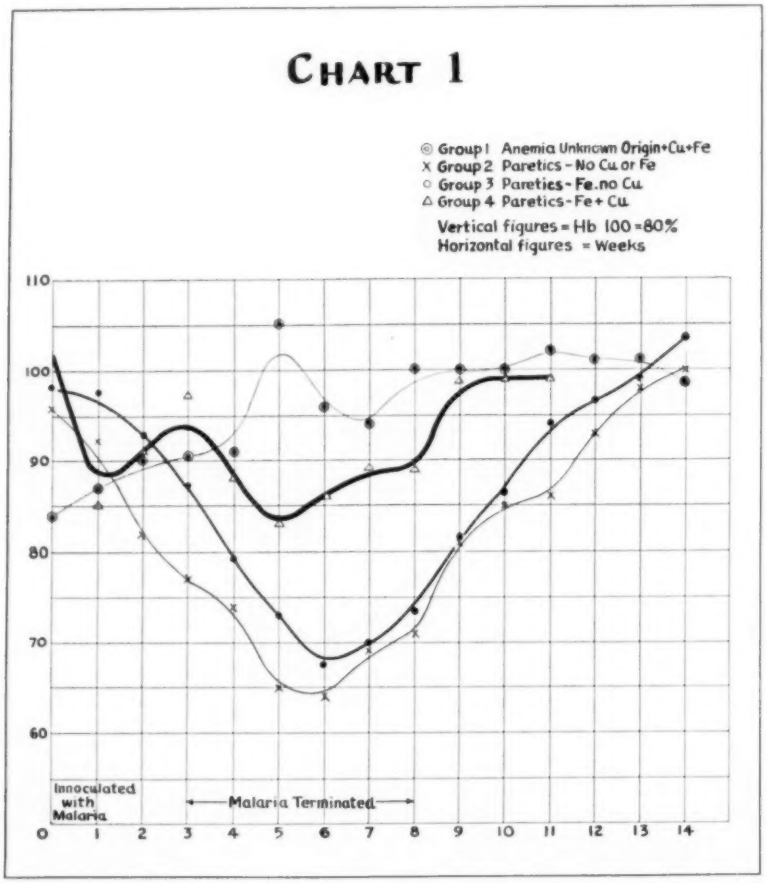
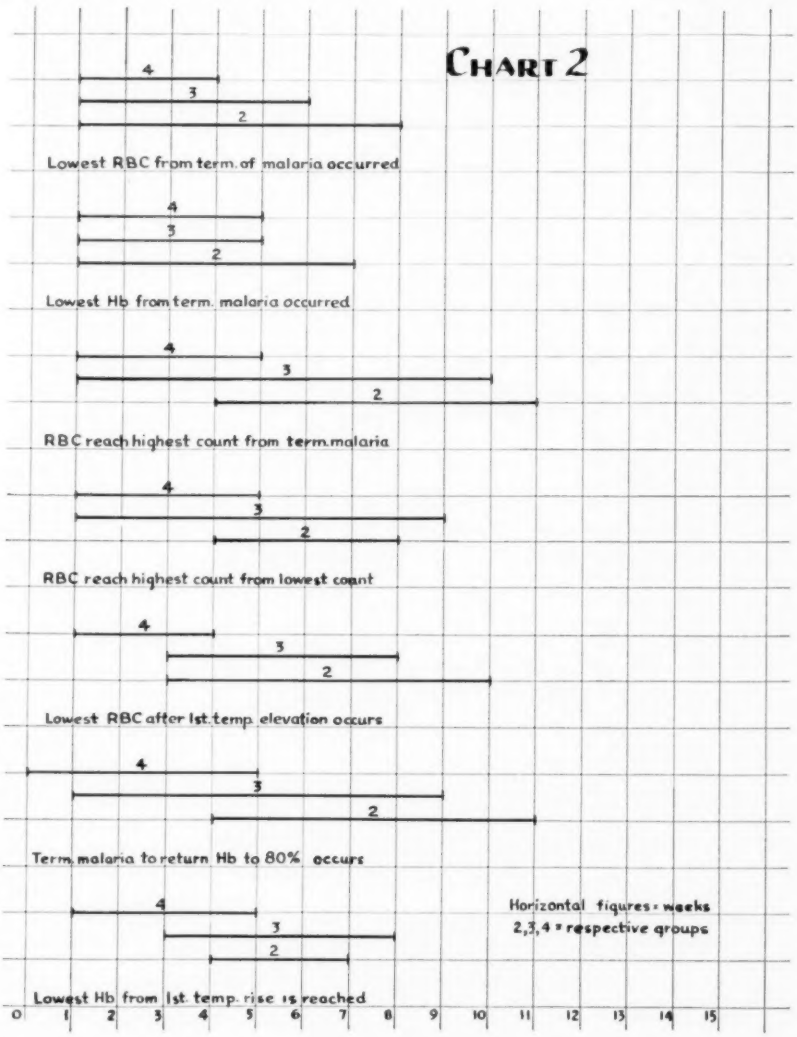


CHART 1
Group 1 Anemia Unknown Origin+Cu+Fe
Group 2 Paretics - No Cu or Fe
Group 3 Paretics - Fe.no Cu.
Group 4 Paretics - Fe + Cu.
Vertical figures = Hb 100 = 80%
Horizontal figures = Weeks
110
100
90
80
70
60
Innoculated with Malaria
Malaria Terminated
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14

istered from the time of the malarial inoculation to the return of the blood picture to normal, the average curve of depression and recuperation of the blood picture as illustrated in the hemoglobin curve in Chart 1 was quite different from those of groups 2 and 3. The curve dropped slower, and before it had dropped half of the distance of those of group 2 or 3, it began to rise again and reached normal far more rapidly than those of either group 2 or 3. As is shown in Chart 2 the time from the first temperature elevation to the lowest hemoglobin and red cell counts was usually shorter, and the return to normal was generally more rapid. This lessened severity of the anemia coupled with the fact that it was more difficult for the malaria to produce the

anemia and the fact that the red blood cell counts and hemoglobin were rapidly restored to normal by means of copper and iron, would indicate that these chemicals are of value in combating the anemia incurred by malaria. There was no evidence that they interfered in any way with the therapeutic benefit derived from the malaria; nor did these drugs interfere with the temperature rises in any way. The copper and iron, furthermore, did not interfere with the action of tryparsamide when this latter drug was administered.



We also feel that they can be used with impunity in dosages much larger than the ones used in this study. It is highly probable that such larger dosages would be more satisfactory than the smaller ones we have used.

Our work indicates, moreover, that the reticulo-endothelial system of man can be stimulated to such an extent that it can minimize the devastation wrought in the blood stream by malaria. It also indicates that the malaria did not materially affect the reticulo-endothelial system of the cases studied and thereby intensify the anemia produced by direct blood destruction.

SUMMARY

A study of the effects of copper and iron on the secondary anemia of therapeutic malaria in general paresis is presented. The results would indicate that these two drugs are of value in lessening the severity and duration of this type of secondary anemia.

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STUDIES IN BONE MARROW*

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THE bone marrow is one of the mysterious and intriguing organs or tissues in the human body. In its significance it may be compared to the central nervous system. There are some similarities and also striking differences between these two tissues. Nature must have considered both very important when it protected them with a hard, resistant, skeletal system—the only two tissues in the body which are enclosed within a bony casing. Both tissues show a quick response to any injury or danger involving the general organism. Oftentimes the bone marrow will show changes, as evidenced by the circulation, long before any other evidence is apparent. On the other hand, while the central nervous system is the most highly specialized tissue in the body, the bone marrow is the most embryonic tissue, and is the only tissue in the body which is persistent as such throughout life, with the exception of such elements as the splenic pulp and several other minor foci of the reticulo-endothelial system. The bone marrow in contradistinction to all fixed tissues, is a potentially wandering tissue in that its elements, which are the product of its activity and proliferation, are the future wandering cells, scattered throughout the circulation and tissue spaces in all parts of the body.

Up to a very short time ago only tissue pathology of the hematopoietic system has been studied intensively, cellular pathology having been considerably neglected since the days of Virchow. To Sabin and her co-workers is due a good deal of the credit for the revival and development of the supravital method of study, this method having been dormant for many years since its inception at the beginning of this century. Her work accomplished in this field is monumental and has helped greatly in the revival of the study of cellular pathology, about which so much is heard now, especially from the standpoint of the reticulo-endothelial system and hematopoiesis. It is from this latter standpoint that this work was undertaken. The elements of the marrow rather than its histologic

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structure was the aim of this study, and the investigation consisted of a study of the quantities of the different types of cells per unit volume of bone marrow, the relative proportion of the various elements to one another, and the character of the different cells in their various stages of differentiation. Beginning with the different types of cells as they are found in the circulation, classified and unclassified, which types are more common and abundant in the bone marrow where earlier forms are discernible, it was hoped to follow these elements in their various stages to their origin in the forms of the primordial blood cells.

The difficulties encountered in the study of the bone marrow may best be summarized by quoting some workers in this field (Doan and Zerfas, J. Exper. Med. **46**: 531, 1927). "Any attempt at a quantitative estimation of the various cellular elements making up the bone marrow must, in the very nature of the survey, always be open to many questionings: the limitations inherent in any technic used, the fallacies inescapable in trying to draw any general deductions from even 1,000 or 2,000 cells counted out of the multiple millions present in any functioning bone marrow, the much debated questions of identification and classification of immature forms, the many factors known and unknown affecting hemopoiesis in any particular individual; all of these and more make it necessary to be conservative in the drawing of deductions from any one limited series of observations or in trying to compare the figures obtained from different investigators."

Accordingly, this study is presented merely as interesting data, observations, and method of procedure. Use was made of material as it became available at the post-mortem table, without reference to any particular type of disease or group of cases. The investigation included all ages and ranged from birth to extreme old age, with a variety of diagnoses in every stage of life.

The same principle was used as that for counting the cells in the circulation, an acetic acid solution for the nucleated elements, and a citrate solution for the red cells as well as all elements. The method may be termed "The Dilution Method." In all cases only rib marrow was used for the quantitative estimation. In most cases one part of marrow was diluted in nine parts of 3 per cent sodium citrate solution, and 2 per cent acetic acid solution, thus giving a suitable dilution for counting, and a convenient factor for calculation. Some cases required a modification of this proportion.

METHOD OF PROCEDURE

Special tubes, 7.5 cm. in length by 7.5 mm. in diameter, were used. These tubes were graduated in tenths of a cubic centimeter. One of these tubes contained 0.9 c.c. of 2 per cent acetic acid solution, and another tube 0.9 c.c. of 3 per cent sodium citrate solution. To each of these tubes were added 0.1 c.c. of bone marrow, and the tubes were shaken gently for about fifteen minutes. The separation of the marrow fat and its rising to the surface of the solution not only did not interfere with the counting process, but aided it considerably in that the counting chamber was almost free from fat particles. The few cells which were enmeshed in the fat did not make any appreciable difference in the results. The dilutions were then drawn up into the proper blood counting pipettes to the 0.5 or 1 mark, as the case required, depending upon the con-

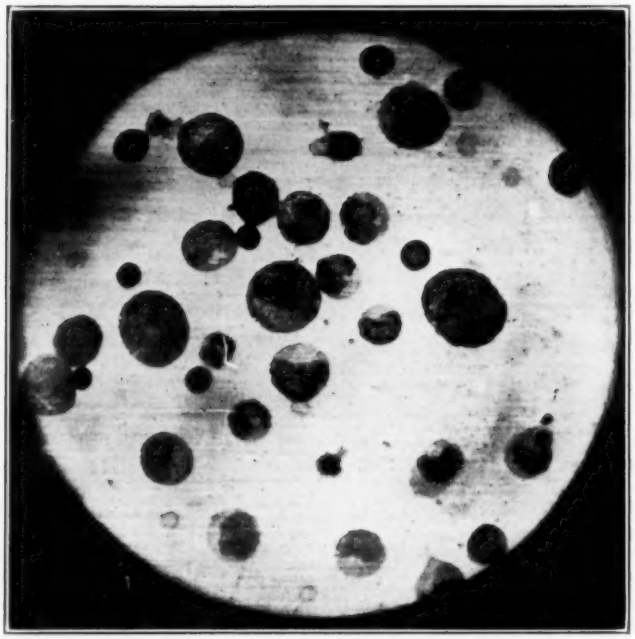

Fig. 1.—Bone marrow smear from a case of myeloid leucemia. Note the proportion of the myeloid, metamyeloid, and the mature polymorphonuclear cells.

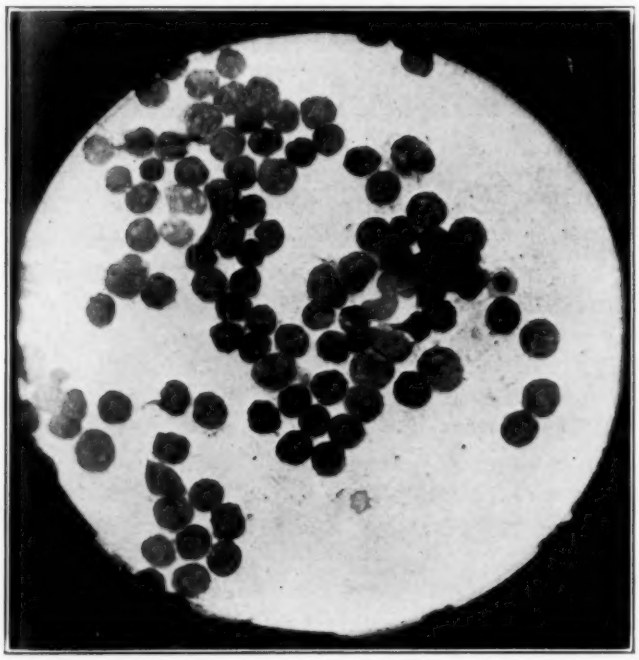

Fig. 2.—Bone marrow smear from a case of lymphoid leucemia. Note the proportion to the myeloid cells.

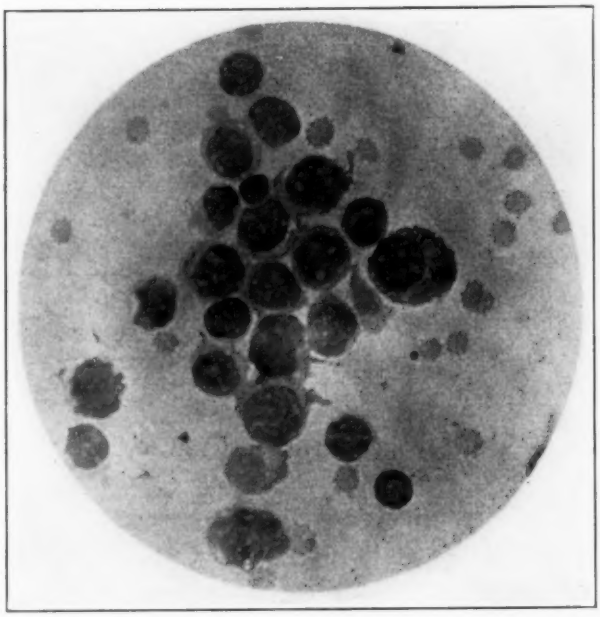

Fig. 3.—Bone marrow smear from a case of myeloblastic leucemia.

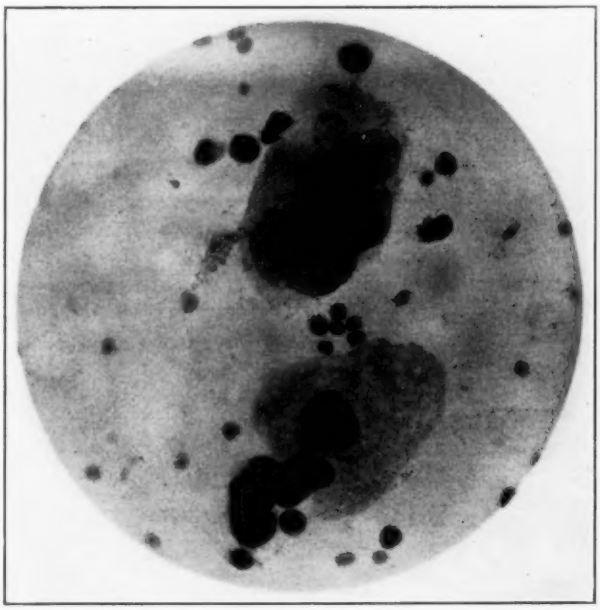

Fig. 4.—Smear of bone marrow showing megakaryocytes. Note their comparison with the other bone marrow elements.

sistency of the marrow, and the rest of the pipette filled with the appropriate solution. The pipettes were again shaken for about ten minutes, and the counts made in the Neubauer hemocytometer. The calculations were made accordingly.

In all cases the total number of all cells and the total number of nucleated cells were counted separately from the different pipettes in different counting chambers. Then the total number of red cells and the total number of nucleated cells were counted separately in the same chamber of the citrate solution, and the two independent figures for the nucleated cells from the two different solutions were compared. In no case were the final figures recorded unless the two separate figures corresponded, allowance having been made for the average technical error. All nucleated cells were counted in one group, comprising all myeloid cells; all nucleated red cells, including normoblasts, megaloblasts, and

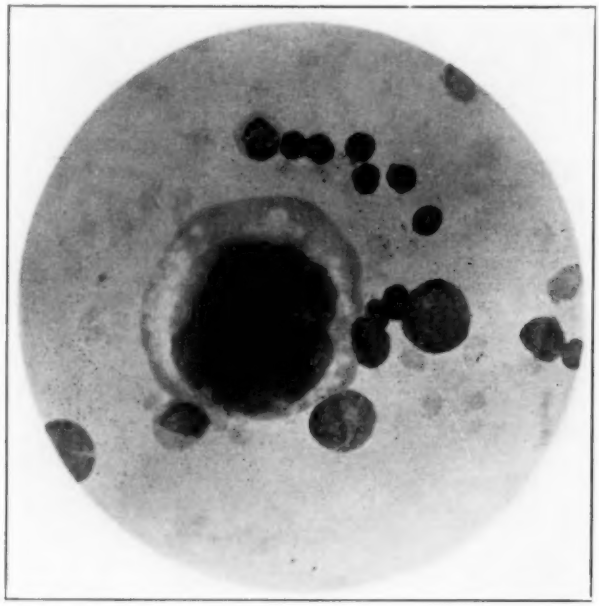

Fig. 5.—Another type of megakaryocyte with a smooth and vacuolated cytoplasm.

still earlier forms of the erythroid series; all lymphoid cells, as well as endothelial cells and all forms of unclassified cells. In some cases, when indicated, the megakaryocytes were counted separately. The further subdivision of these cells becomes a problem for differential study of the stained smear, for attempts were not made to differentiate the various types of cells in the counting chamber. The results obtained are presented in tabular form, giving the sex and age of the patient, the clinical and pathologic diagnosis, the number of red blood cells and nucleated blood cells per cubic millimeter of blood as found in the circulation a short time before death, the red blood cells and nucleated cells of all types as found in the bone marrow per cubic millimeter of marrow.

DIFFERENTIAL STUDY

The same "dilution principle" was used as that for the quantitative estimation. However, for the differential study blood serum was used for dilution. Autogenous serum, of course, is ideal, but this was not always procurable.

Therefore, the blood group of the individual was determined from the marrow, and corresponding serum was used for dilution. The proportion of serum to bone marrow cannot be fixed for use in all cases, as each marrow requires an amount commensurable with its own physical characteristics, and the proper quantity to be used must be judged from experience. Sometimes several mixtures must be made before obtaining a proportion which would give the proper smears. The Romanowsky dyes were used for staining. In some cases, such as myeloblastic leucemia, lymphoid leucemia, agranulocytic angina, aplastic anemia, or any other forms of severe anemia of unknown etiology, the results were counterchecked with the supravital stain and the oxydase stain. Satisfactory results were obtained in most cases, except those in which the autopsy material was not fresh, or when the autopsy was done a long time after death.

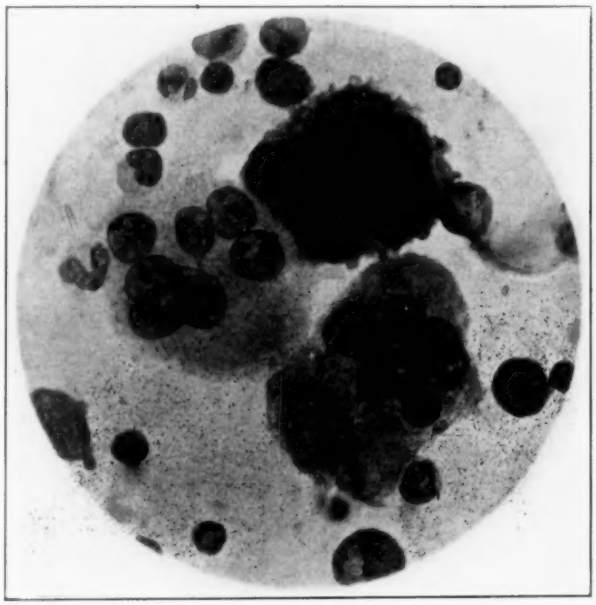

Fig. 6.—Three megakaryocytes in one field. This smear was made from a bone marrow of a case of myeloid leucemia.

Such unsatisfactory material was discarded after attempts to study them, and the results omitted from the record.

DISCUSSION

An analysis of Table I is tempting of many comments. To begin with, the proportion of nucleated cells to red blood cells in the circulation normally is about 1:700, while in the bone marrow the proportion drops considerably, reaching almost 1:1, and in many cases the nucleated elements are more abundant than the red cells. This is probably due to the fact that the red cell has a rather long life and a great storage of these elements is not necessary, while the short-lived white cell requires an abundant storage supply for a constant and rapid turnover. After studying many pathologic bone marrows, the question arose as to what is the normal cellular content of the bone marrow per unit volume. The opportunity for such observation presented itself in Case 23. A young healthy male, 28 years old, was working on a scaffold repairing the outside of a

TABLE I

NO.	SEX	AGE	DIAGNOSIS	R.B.C. IN CIRCULATION	R.B.C. IN BONE MARROW	NUCLEATED CELLS IN CIRCULATION	NUCLEATED CELLS IN BONE MARROW
1	M	2	Otitis media. Meningitis	4,020,000	2,130,000	22,000	920,000
2	F	33	Addison's disease	4,320,000	4,100,000	18,000	1,480,000
3	M	62	Acute myeloblastic leucemia	2,300,000	695,000	19,000 (3d) 3,800 (1d) 400 (5h)	220,000
4	F	34	Endocarditis. Terminal pneumonia	4,180,000	840,000	18,600	1,200,000
5	F	48	Diabetes. Terminal pneumonia	4,180,000	1,125,000	19,200	1,024,000
6	M	8	Aplastic anemia	2,120,000	110,000	750	28,000
7	M	33	Myeloid leucemia	2,850,000	392,000	96,000 (5d) 128,000 (3d) 130,000 (1d)	1,024,000
8	M	70	Cardiac decompensation	4,160,000	704,000	8,100	512,000
9	M	59	Hypernephroma	3,600,000	1,152,000	11,800	416,000
10	M	8 mo.	Sepsis	4,120,000	2,464,000	40,000	968,000
11	M	68	Aneurysm. Syphilis	4,900,000	1,072,000	10,000	512,000
12	F	45	Cardiac decompensation	4,320,000	488,000	6,500	672,000
13	M	6	Lung abscess	2,430,000	1,464,000	15,300	960,000
14	M	63	Carcinoma of pancreas	3,700,000	648,000	11,800	384,000
15	M	2	Sepsis	4,120,000	680,000	16,300	412,000
16	M	1	Syphilis. Pneumonia	3,100,000	1,160,000	33,000	1,024,000
17	M	5 mo.	Otitis media. Bronchopneumonia	6,150,000	5,792,000	9,400	288,000
18	M	3 mo.	Otitis media. Meningitis	4,460,000	1,056,000	14,800	472,000
19	M	64	Coronary sclerosis	5,440,000	558,000	7,100	604,000
20	F	52	Agranulocytic angina	4,900,000	1,120,000	1,100	224,000
21	M	45	Lung abscess	3,810,000	1,112,000	13,400	408,000
22	M	73	Adenocarcinoma of left kidney	4,350,000	1,352,000	9,500	480,000
23	M	28	Traumatic shock		1,132,000		468,000
24	M	17	Acute nephritis	4,560,000	720,000	11,400	788,000
25	M	7 mo.	Sepsis	7,550,000	2,218,000	10,650	240,000
26	M	2 wk.	Bronchopneumonia	4,660,000	2,320,000	30,600	536,000
27	F	2 mo.	Rickets. Sepsis	4,300,000	1,096,000	30,000	1,024,000
28	M	56	Polycythemia	13,000,000* 10,760,000* 5,200,000*	1,168,000	29,000 (3m) 16,000 (2m) 13,400 (3d)	512,000
29	M	50	Myocarditis. Terminal pneumonia	4,050,000	912,000	11,000	788,000
30	M		Stillbirth		2,120,000		576,000
31	M	39	Agranulocytic angina	4,440,000	672,000	200	208,000
32	M	24	Aplastic anemia	850,000	336,000	2,600	80,000
33	M	56	Lymphatic leucemia	1,664,000* 984,000*	192,000	233,600 (5d) 342,000 (1d)	1,320,000

m = months before death
d = days before death
h = hours before death
* = same time as nucleated cells in circulation

building. He lost his balance, slipped, and fell off the scaffold, striking the concrete sidewalk underneath. He was rushed to the hospital at once, but he died soon after reaching it. A post-mortem examination was done within a half hour after the accident occurred, and the bone marrow showed a red cell count of 1,132,000, and a nucleated cell count of 468,000 per cubic millimeter. There was no history of previous illness, and the man was said to be in excellent health since childhood. Histologic studies of all organs and tissues showed no pathologic changes. The findings then are assumed to be those of a normal healthy individual. It is more interesting, therefore, to compare these findings with those seen in Case 21, with the diagnosis of lung abscesses of several months' duration following an acute pneumonia, or with the findings in Case 22, with the diagnosis of adenocarcinoma of the kidney (see also Cases 9 and 18). One probable explanation may be that whatever the original stimulus and its resultant bone marrow response, when the disease had progressed for many months or longer, either the stimulus or the mechanism of response, or both, were exhausted, and the bone marrow in time began to assume a static position in its activity. Frequently evidence in the circulation tends to corroborate this view.

In other cases, the bone marrow findings may be misleading without an adequate and detailed study from other angles. This is particularly true in some of the blood diseases. In Case 7, a case of myeloid leucemia, in which the nucleated cell count in the circulation is over 100,000 per cubic millimeter, the nucleated cell count in the bone marrow does not reflect the unusual proportion, but merely shows a count two or three times the average normal amount. However, when one takes into account the tremendous increase in the total volume of marrow, as shown by the large size of the rib, with almost a spherical diameter, and the densely packed succulent marrow, the proportion is seen to be much greater than it appears at first glance. One may also speculate at the role played by the endocrine system in the change of the skeletal structures containing the marrow. Surely the change in the size of the bony tissue must have been going on for a long time before the leucemic state made its appearance or was recognized clinically. This change in the rib structure is not seen in lymphoid leucemia, probably because of the infiltrative rather than proliferative character of this type of leucemia in the bone marrow, and the displacement of the red cell elements by this infiltration. Another point of interest is the minimum or lowest number of nucleated cells in the marrow seen in such cases as aplastic anemia, agranulocytic angina, or certain cases of myeloblastic leucemia. When the bone marrow reaches a certain low level, no nucleated cells will trickle through into the circulation. These and many other phases of bone marrow activity tempt one to speculate as to the interpretation of these findings in the hope of correlating them with clinical applicability.

SUMMARY

1. A method is presented for the quantitative estimation of the cellular elements of the bone marrow and for differential study.

2. By this method results were obtained which promise an approach to the study of bone marrow cytology from new angles.

61 Eastern Parkway

LETHAL DIFFERENCES BETWEEN PANCREATIC EXTRACT (WOLFFE) AND ACETYL-BETA-METHYLCHOLINE IN ATROPINIZED ANIMALS*

VICTOR A. DIGILIO, M.D., JOSEPH A. PESCATORE, M.D., AND HAROLD E. GOLDBERG, M.D., PHILADELPHIA, PA.

EXTRACTS of animal tissues have long been known to contain impurities such as histamine and choline. Objections have been raised against pancreatic extracts on these grounds. As a matter of fact, it has been intimated that the therapeutic efficacy of these extracts may be due to the impurities.

Wolffe and Munch[1] showed that Norit-treated pancreatic extracts retained their potency and did not lose their heart-blocking properties. Moreover, they added known quantities of histamine to the solutions and were able to recover practically 100 per cent by this method. It has also been shown[2] that atropine prevents the depressor action of acetylcholine, but fails to prevent the depressor action of pancreatic extract. In spite of this, criticism still exists. Since both substances when administered in adequate dosage cause heart block in animals, and since atropine is pharmacologically antagonistic to acetyl-β-methylcholine, another method presents itself for the detection of any differences in action between pancreatic extract (Wolffe) and acetyl-β-methylcholine. If the former substance owes its activity to the presence of choline impurity, it should be expected to act in a fashion similar to the action of any pure choline substance.

METHOD

Choline impurities in pancreatic extract are an unknown quantity. In order to compare these substances in animals it was obviously necessary to ascertain the smallest amount of each substance which would consistently cause heart block and death in white mice, of a known strain, weighing between 24.3 and 25.8 gm. each. It was found that 1 c.c. of pancreatic extract made according to Wolffe[3] and standardized according to the published method[4] (so that 1 c.c. of extract possessed 20 gamma epinephrine neutralizing units) caused death by intraperitoneal injection within five to nine minutes in 9 out of 10 animals (the last animal died of intra-abdominal hemorrhage). It was observed that the animals consistently developed heart block, but that respiration ceased while occasional ventricular systole could still be registered electrocardiographically for several seconds afterward. It was likewise determined that 7 mg. of acetyl-β-methylcholine (7 mg. per c.c.) by intraperitoneal injection caused heart block and death in 10 animals. In this group

*From the Temple University Hospital.
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the heartbeat and respirations ceased simultaneously within six to nine minutes. Smaller doses of either substance caused lesser degrees of cardiac slowing but seldom death.

A group of 20 white mice of the same strain and weight as the above were divided into subgroups of 5 each. All the animals were kept under the same conditions of temperature, feeding time, etc. All experimental procedures were carried out at the same time of the day, and each animal was

Protocol I

Lethal Dosage of Acetyl-β-Methylcholine Chloride*

Mouse No. 273	male—25.3 gm.		
	2:19— 7.0 mg. acetyl-β-methylcholine	8	minutes
	2:27—animal dead		
Mouse No. 275	male—24.5 gm.		
	3:05—P.M. 7.0 mg. acetyl-β-methylcholine	6½	minutes
	3:11½—animal dead		
Mouse No. 276	male—25.1 gm.		
	3:30— 7.0 mg. acetyl-β-methylcholine	7¼	minutes
	3:37¼—death		
Mouse No. 277	male—25.6 gm.		
	3:41— 7.0 mg. acetyl-β-methylcholine	9	minutes
	3:50—death		
Mouse No. 278	male—24.4 gm.		
	3:53— 7.0 mg. acetyl-β-methylcholine	8	minutes
	3:59—death		

*Acetyl-β-methylcholine was supplied by Merck & Co., as the chloride.

Protocol II

Effect of Pancreatic Extract on Mice

Mouse No. 285	male—25.5 gm.
	3:12—1 c.c. pancreatic extract (Wolffe)
	3:15—animal breathing slowly and irregularly
	3:17½—animal dead
Mouse No. 286	male—24.7 gm.
	3:19—1 c.c. pancreatic extract (Wolffe)
	3:27—animal dead
Mouse No. 287	male—24.3 gm.
	3:30—1 c.c. pancreatic extract (Wolffe)
	3:35—animal dead
Mouse No. 288	male—25.4 gm.
	3:36—1 c.c. pancreatic extract (Wolffe)
	3:43—animal dead
Mouse No. 289	male—25.1 gm.
	3:46—1 c.c. pancreatic extract (Wolffe)
	3:52—animal dead

NOTE: All mice, Nos. 285-289, died after having a convulsive spasm, during which their limbs became rigid, bending toward each other, the body as a whole becoming very taut, and the tail rising almost perpendicularly. After a few seconds in this position, the body relaxed, the tail came down, and the animal ceased breathing.

weighed at the beginning of the experiment. Animals under 24.3 gm. or over 25.8 gm. were discarded. The subgroups were arranged as follows:

Group 1: received acetyl-β-methylcholine
Group 2: received pancreatic extract
Group 3: received acetyl-β-methylcholine after atropinization
Group 4: received pancreatic extract after atropinization

All injections were made by the intraperitoneal route. One cubic centimeter volumes were employed in each injection, so that 1 c.c. of acetyl-β-

Protocol III

Atropinization of Mice and the Subsequent Administration of Acetyl-β-Methylcholine

Mouse No. 280	male—24.6 gm.
	3:07— 0.013 mg. of atropine sulfate injected
	3:22— 7.0 mg. of mecholin injected
	3:28—animal sluggish, respiration and heart rate slow
	3:40—animal still sluggish
	3:52—animal recovering slowly; heart rate more rapid but still not at control level
Mouse No. 281	male—25.1 gm.
	3:31— 0.013 mg. of atropine injected
	3:46— 7.0 mg. mecholin injected
	3:58—animal less active; slower heart rate
	4:10—animal active; heart rate approaching control rate
Mouse No. 282	male—24.5 gm.
	3:50— 0.013 mg. of atropine sulfate injected
	4:05— 7.0 mg. of mecholin injected
	4:10—animal very sick; slower heart action
	4:14—respiration very slow and irregular
	4:21—respiration regular but slow
	4:30—animal started to walk; heart rate more rapid
Mouse No. 283	male—25.5 gm.
	4:18— 0.013 mg. atropine sulfate injected
	4:33— 7.0 mg. mecholin injected
	4:40—animal slower in action; heart rate slower
	4:44—breathing labored
	4:52—animal fairly recovered; heart action rapid but still at subcontrol level
Mouse No. 284	male—24.9 gm.
	4:41— 0.013 mg. atropine injected
	4:56— 7.0 mg. mecholin injected
	5:05—respiration and heart rate slower
	5:13—animal breathing regularly but slower than normal
	5:25—animal active; heart rate more rapid

NOTE: All the above mice were alive, active, and apparently normal twenty-four hours after the close of the experiment. Heart rates returned to control levels within two to four hours following the administration of acetyl-β-methylcholine and remained so at the end of the twenty-four-hour period.

Protocol IV

Effect of Pancreatic Extract on Mice Which Previously Had Been Atropinized

Mouse No. 290	male—25.3 gm.
	2:05— 0.013 mg. atropine sulfate
	2:20—1 c.c. pancreatic extract
	2:25½-animal dead
Mouse No. 291	male—24.8 gm.
	2:99— 0.013 mg. of atropine sulfate
	3:44—1 c.c. pancreatic extract
	3:51—animal dead
Mouse No. 292	male—25.7 gm.
	3:54— 0.013 mg. atropine sulfate
	4:09—1 c.c. pancreatic extract
	4:17—animal dead
Mouse No. 293	male—24.9 gm.
	4:20— 0.013 mg. atropine sulfate
	4:35—1 c.c. pancreatic extract
	4:42—animal dead
Mouse No. 294	male—24.3 gm.
	4:45— 0.013 mg. atropine sulfate
	5:00—1 c.c. pancreatic extract
	5:06—animal dead

NOTE: These mice died in the same manner as Nos. 285-289.

methylcholine solution contained 7 mg. of drug and 1 c.c. of Wolffe extract contained 20 epinephrine neutralizing units. In each instance electrocardiograms were taken employing hypodermic needles embedded into the limb muscle of the animals and connected to the Lead II wires of an electrocardiograph

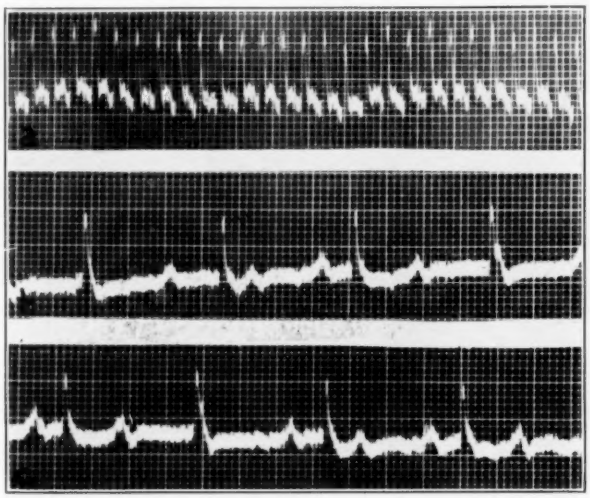

Fig. 1.—Acetyl-β-methylcholine control. *a*, Control tracing; *b*, four and one-half minutes after injection of acetyl-β-methylcholine, showing heart block; *c*, heart block continued and death of the animal occurred shortly afterwards.

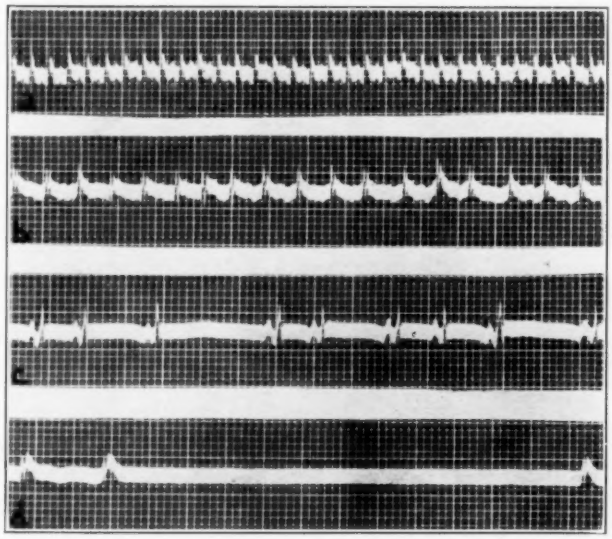

Fig. 2.—Pancreatic extract control. *a*, Control tracing; *b*, slowing of heart rate with beginning inversion of initial ventricular complex; *c*, complete heart block and complete inversion of both ventricular complexes; *d*, death nine and one-half minutes after injection and initial ventricular complex became upright again.

(G. E. portable; right forelimb and left hindlimb) by conductor clips. Tracings were taken before and at intervals after injecting the animals. Atropine sulfate in doses of 0.013 mg. per c.c. was injected into animals to be atropinized, and fifteen minutes allowed for complete absorption before administering the

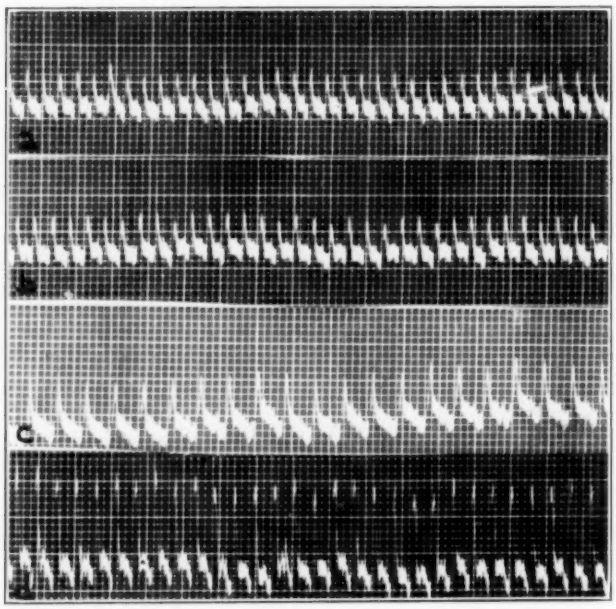

Fig. 3.—Acetyl-β-methylcholine injection after atropinization. *a*, Control tracing; *b*, tracing taken fifteen minutes after atropine administration; *c*, slowing of heart rate approximately ten minutes after injection of acetyl-β-methylcholine; *d*, return to control rate about two hours after injection of acetyl-β-methylcholine.

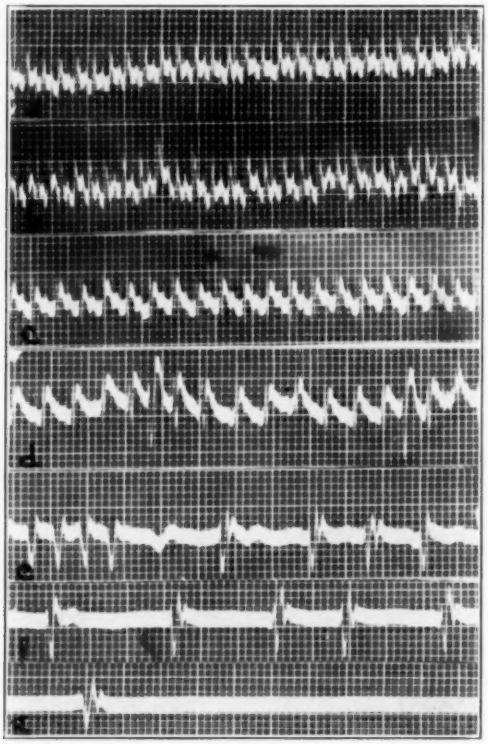

Fig. 4.—Pancreatic extract injection after atropinization. *a*, Control tracing; *b*, tracing fifteen minutes after injection of atropine; *c*, four minutes after pancreatic extract injection; *d-e*, five and six minutes, respectively, following pancreatic extract injection; *f*, heart block and inversion of QRS waves; *g*, death in seven minutes.

experimental substance. Nonatropinized animals received 1 c.c. of physiologic saline in place of atropine and a similar interval was allowed to pass. Nonsurviving animals were autopsied in order to eliminate the possibility of trauma as a factor causing death.

RESULTS

All the animals in both control groups died; all had heart block. Those to which pancreatic extract was administered developed block, arrested respiration while the heart continued to beat for several seconds but convulsions were unusual. Those receiving acetyl-β-methylcholine developed heart block, salivation, and usually died in tonic convulsions. Heart action and respirations stopped simultaneously.

The electrocardiograms of each of the control groups revealed some interesting differences (Figs. 1 and 2): (1) Following pancreatic extract administration there was constant inversion of the initial ventricular complex with re-inversion immediately before cardiac arrest. (2) Ectopic ventricular rhythms, such as ectopic beats and occasionally small intervals of ventricular tachycardia, were observed in the pancreatic animals but rarely in the animals injected with acetyl-β-methylcholine. In the latter group the ventricular rhythm was of the supranodal type throughout.

All the atropinized animals receiving pancreatic extract developed heart block and died (Figs. 3 and 4). Those receiving acetyl-β-methylcholine consistently demonstrated slowed heart rates but by no means to the extent noted in the controls (Fig. 3). All of this group survived. Salivation was more or less constant, but no convulsions were noted. Heart rates were normal from two to four hours after the administration of acetyl-β-methylcholine.

COMMENT

The constancy of the results points to a distinct difference in the substances. While both are parasympathetic stimulants, it is nevertheless true that acetyl-β-methylcholine and pancreatic extract (Wolffe) exhibited dissimilar effects in atropinized white mice. As a matter of fact, the electrocardiographic changes in the controls appeared to be constant for each group and demonstrated real and recognizable differences between these groups. The objection could be raised that since acetyl-β-methylcholine was able to slow the heart action of these animals in spite of atropine, an insufficiency of the latter drug was administered. It would seem to us the objection is not valid because: (1) the experimental conditions, as stated, were equal for all groups of mice; (2) multiples of the stated atropine dosage failed to yield results other than those above described; and (3) previous investigations over the past four years do not support the objection.

SUMMARY

1. Pancreatic extract (Wolffe) and acetyl-β-methylcholine exhibit differences of action in atropinized white mice under the experimental conditions above described.

(a) Pancreatic extract action was not inhibited by atropine.

(b) The lethal effects of acetyl-β-methylcholine were prevented by atropinization.

(c) Atropine did not prevent the complete heart block which pancreatic extract (Wolffe) consistently caused in the controls.

(d) Although bradycardia occurred in the atropinized animals which received acetyl-β-methylcholine, it was not as severe in degree as the complete A-V dissociation seen in the controls.

2. Electrocardiographic differences between the control groups were obviously definite.

(a) The pancreatic animals exhibited inversion of the initial ventricular complexes and ectopic ventricular arrhythmias not demonstrated in the tracings of the animals receiving acetyl-β-methylcholine.

3. Since the dosage of pancreatic extract (Wolffe) employed in these experiments is out of all proportion per body weight to the largest doses used in human beings, the above observations cannot be used as an index of toxicity.

CONCLUSION

The above findings suggest fundamental differences of action between acetyl-β-methylcholine and pancreatic extract (Wolffe). The magnitude of this disparity warrants the statement that the choline substances present in pancreatic extract (Wolffe) do not account for the action of this extract under the conditions of this experiment, unless such substances (choline) possess properties heretofore not attributed to or demonstrated by choline derivatives.

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CORONARY ARTERY THROMBOSIS*

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A RECENT survey of the cases of acute coronary artery thrombosis at the Henry Ford Hospital has been carried out in an attempt to evaluate the experiences of the past in the light of present knowledge. Since the installation of our electrocardiographic instrument in 1923, there have been 159 cases of undoubted coronary occlusion in this hospital. One hundred eleven patients were successfully traced, and of these 83 were dead; 46 of the latter number had been examined post mortem. The reclassification of the cases was done with the plan of Wilson and co-workers, dividing them by means of electrocardiographic changes into anterior, posterior, and indeterminate groups. The material at hand has been grouped as in Table I.

Table I

Cases Studied

Total cases	159
Studied with EKG	146
Cases traced to date	111
Cases traced—still living	28
Cases traced—died	83
Autopsied	46
Cases dead in first year	60 (54 per cent)

This material was critically selected from perhaps twice that number of cases indexed as coronary thrombosis. Those which, for one reason or another, failed to answer a careful inquiry were cast out, and the resulting group is felt to be that of proved cases only.

Pertinent factors, such as age, sex, race, and occupation, and of the clinical aspects, such as the number of cases showing fever, leucocytosis, pericardial friction rub, arrhythmias, etc., were not different from previously published series of cases. The monograph of Levine[2] and the recent book edited by Levy[3] contain adequate statistical information regarding these factors. In the present series the youngest case was 29 and the oldest 79 years, while the average age of the entire group was 55.8 years. The decade of greatest incidence was from 60 to 69 years. The sex ratio was 5.36 males to 1 female. In the absence of syphilis the disease was not found to occur in colored persons in this series. Definite obesity was more frequently present than absent. No equitable classification of occupation could be made from a survey of our records, because the factors of pressure, emotional tension, and nervous stimuli are so variable

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in all types of activity. It is interesting to record that only 47.7 per cent presented a story of previous angina of effort. Eighty-one and seven-tenths per cent of the group gave a history of crushing, constricting, viselike pain. In 77 per cent of all cases there was continuous pain of longer than four hours' duration. The number would undoubtedly have been higher, except that many of the patients were relieved by hypodermic medication. Vomiting occurred in more than one-fourth of the patients, and prostration was a frequent manifestation.

The clinical observations on patients suffering from acute coronary artery thrombosis characteristically include fever, leucocytosis, and fall in blood pressure. In somewhat lessened frequency are found gallop rhythm, cardiac arrhythmias, and pericardial friction rub. The changes in the blood sedimentation rate appeared to persist longer than the other evidence of acute tissue damage.

In this report, however, the primary interest is in the accuracy of the electrocardiographic information as to the presence of myocardial infarction and the localization of the infarction if present. A similar study has been previously published by Sprague and Orgain.[7] The question of electrocardiographic localization of myocardial infarction was given its initial clinical impetus by Barnes and Whitten,[4] their work being based, in part, on a previous contribution by Parkinson and Bedford.[5] The latter authors had differentiated electrocardiograms of the T_1 and T_3 types, while Barnes and Whitten demonstrated the relationship of T_1 curves to anterior and T_3 to posterior infarction. Later Wilson and co-workers[1] noted the frequency of Q-wave prominence in the two major types of infarction, and elaborated this concept into electrocardiograms of the Q_1T_1 and Q_3T_3 types. In the present study the standard three lead electrocardiograms were styled Q_1T_1 type, indicating an anterior infarction by left coronary artery thrombosis, or Q_3T_3 indicating a posterior infarction by right coronary artery thrombosis.

Fig. 1 outlines the plan ordinarily used in this clinic to differentiate the various locations of infarction. This figure represents the presence of the primary $S.T._1$ elevation with subsequent evolution into Q_1T_1 contour suggesting anterior infarction. Infarction in the posterior wall is anticipated when the primary S.T. elevation occurs in Lead III.

On analysis of our electrocardiograms, some were clearly examples of anterior infarction, some were clearly posterior, while a third group, just as certainly made up of acute coronary artery thrombosis, did not fit into the two clearly differentiated groups. These have been styled indeterminate types of coronary occlusion. Some of them show only small complexes; others have various conduction disturbances, such as auricular flutter, which vitiate any attempt to localize the area of damaged heart muscle.

Of the 146 cases classified according to the above plan, 111 have been traced. Eighty-three of these are dead and 28 have survived. In Fig. 3 are seen the survival periods of the cases whose histories are finished, that is, the dead cases.

It will be noted that the greatest mortality was in the first few months, regardless of the type of electrocardiographic abnormality. Thus, in Fig. 2 it will be noted that of the anterior group, 51 per cent were dead in the first year,

while 45 per cent of the posterior group had a similar fate. The indeterminate group had a somewhat higher first year mortality (70 per cent), perhaps because many of them were of the small complex, or conduction disturbance type. It is noteworthy that in the 83 patients who died, prognostic implications of electrocardiograms of the Q_1T_1 and of the Q_3T_3 type are very similar. This is in op-

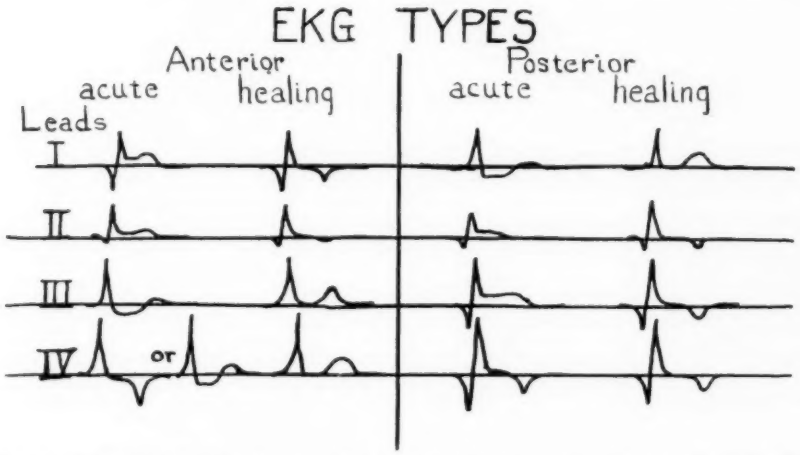

Fig. 1.—Chart of usual electrocardiographic changes met with in the anterior and posterior types of coronary artery thrombosis illustrating basis of differentiation made in this study. Axial leads in anterior infarction show major changes in Lead I tending to the Q_1T_1 type of curve. Posterior infarction is indicated by major changes in Lead III tending toward the Q_3T_3 type. Alterations in Lead IV prevail in anterior infarction.

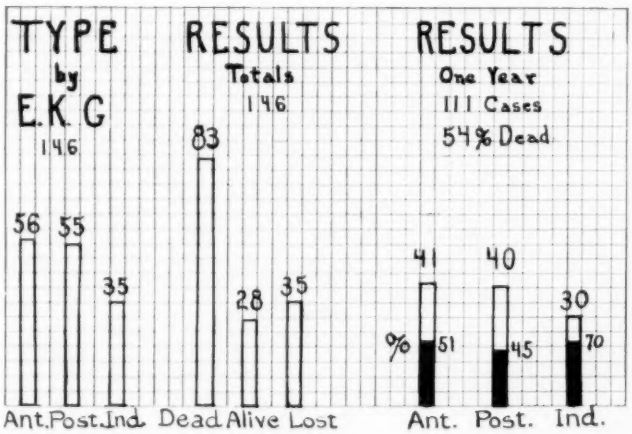

Fig. 2.—Illustrating the relative proportion of anterior, posterior, and indeterminate forms of coronary artery thrombosis as indicated by EKG changes. Also shown are the mortality rates in the first year for the three classes of thrombosis. It will be noted that anterior and posterior infarction carry almost the same first year mortality.

position to the view expressed by Wood and associates,[6] who felt that the immediate and future prognosis of the posterior, or Q_3T_3, group was less grave. In our experience the posterior group demands the same conservatism in prognosis as does the anterior group.

The preceding discussion as to electrocardiographic differentiation and survival period has dealt largely with the clinical aspects of the condition. The result of this survey forces the conclusion that in a few cases the electrocardiographic

changes and the autopsy findings will not be in strict agreement. Thus, of 16 fresh single infarctions, localization by the standard three lead electrocardiograms was accurate in only 5 instances. It gave definitely misleading information in 3 cases. Of the 8 doubtful cases, all having anterior infarctions, 1 showed no detectable abnormality, 4 were indeterminate, and 3 suggested posterior infarction. Hence, localization of cardiac infarction, using only the standard 3 leads, is frequently impossible and may lead to false conclusions.*

With this thought in mind, 46 autopsied cases of coronary thrombosis have been studied (Table II). Of these, 31 were studied with one or more electrocardiograms. Since it was realized that cases showing marked coronary artery

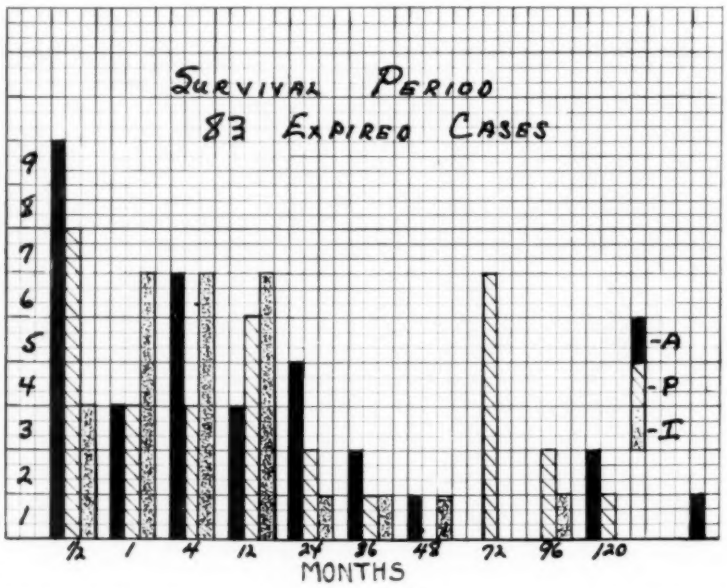

Fig. 3.—Illustrating the survival period of 83 dead cases. *A*, the anterior occlusions as indicated by EKG changes; *P*, the posterior; and *I*, those with changes of indeterminate origin.

sclerosis and multiple infarctions might show confusing pictures in the electrocardiogram,[8] the cases were divided into those with single fresh infarcts and those with associated additional pathologic changes. Sixteen cases of fresh single in-

TABLE II

AUTOPSIED CASES

Total	46
With EKG	31
Single fresh infarct—anterior	13
Single fresh infarct—posterior	3
Old and recent infarcts	8
Old without recent infarcts	6
Sclerosis—infarct—no occlusion	1

*The autopsy protocols have been dictated by several pathologists over a number of years. Personal inclination and interest have caused a variable degree of thoroughness in describing the pathologic changes. The usual autopsy routine may well fail to reveal all areas of previous infarction or occlusion of minor coronary branches. This objectionable feature has also been commented upon by Sprague and Orgain.

farction were studied in detail. Thirteen were of the anterior type at autopsy and 3 of the posterior type. We have attempted to correlate the electrocardiograms obtained in these cases with the actual location of the infarction. Using this method, a majority of the curves were found to be of surprisingly little help, or of questionable help in the actual localization of the infarction as demonstrated at autopsy (Table III).

TABLE III

EKG LOCALIZATION OF 16 CASES OF FRESH SINGLE INFARCTION

Standard 3 Leads Only

EKG	ANTERIOR INFARCTION	POSTERIOR INFARCTION
Consistent	3	2
Doubtful	8	
Inconsistent	2	1

Table IV summarizes the 16 cases of fresh single infarctions.

It is remarkable to note that patients showing marked myocardial destruction, leading in some cases to rupture, could show only minor changes in electrocardiograms obtained shortly before death. Thus, in Case 3, the electrocardiogram of May 22, 1925, was normal, but the patient died on May 30, 1925, of a ruptured myocardium. Some of these curves studied critically with no knowledge of the actual location of the infarction, might be fairly stated to resemble the Q_3T_3, or posterior type of infarction, while the autopsy disclosed anterior infarction instead. It is appreciated that in many cases one electrocardiogram will not suffice and that progressive changes from day to day are frequently more significant. These records point to the need for caution in the interpretation of single electrocardiograms. No case showing typical Q_1T_1 or anterior infarction change in the electrocardiogram was proved otherwise at autopsy.

Since 1935, we have been using the precordial leads as outlined by Wood and Wolferth and by Wilson. The technique of obtaining Lead IV has been standardized by an international committee,[9] and since January, 1938, the recommended method F-4 has been followed in this clinic. However, in the material presented here Lead IV was obtained by a now discontinued method. That is, the right arm electrode was placed over the precordium. We were accustomed to locate this electrode at both the cardiac apex and in the left fourth interspace at the sternal border. The indifferent electrode was placed in the left interscapular region. Thus in these records a prominent Q_4 and an inverted T_4 are considered to be normal.

Of the 16 instances in which autopsy disclosed single fresh infarction, 5 were studied with the precordial lead (Lead IV), as well as with the standard leads. In 3 of these, Lead IV (Cases 9, 10, and 13) gave important evidence not available in the axial leads. In these 3 cases, Q_4 was absent, and in 2 of them, in addition, the T-wave was upright. In the remaining 2 records Lead IV was suggestive only. One of them showed depressed ST_4, though Q_4 was present, while in the other, Q_4 was likewise present but the T-wave was upright.

Hence the information obtained with Lead IV was not misleading in any instance. On the contrary, in those cases where the axial leads were not in-

TABLE IV

CASE NO.	PAIN	EKG	AUTOPSY
1	7/13/32	7/19/32 Small Q_2 and Q_3 Indeterminate	7/27/32 Infarction—left ventricle and septum Vessels—left descending occluded; right, marked sclerosis
2	11/14/28	11/16/28 S.T. interval depressed all leads Small Q_2 and Q_3 Indeterminate	12/ 1/28 Infarction—apex at left ventricle Vessels—ostia left not visible; right, pin point; caliber vessels open
3	5/ 8/25	5/22/25 Normal	5/30/25 Infarction—apex left ventricle with rupture Vessels—left descending occluded; right, no obstruction
4	3/24/30	4/ 5/30 S.T. interval depressed Lead I, II, elevated in III Prominent Q_3 Suggests posterior	6/ 6/30 Infarction—apex left ventricle and septum Vessels—left, descending occluded; right, no obstruction
5	6/16/28	6/18/28 S.T. interval depressed Lead I, elevated in III Prominent Q_3 Inconsistent	6/20/28 Infarction—anterior wall left ventricle Vessels—left circumflex occluded; right, no obstruction
6	3/22/29	4/ 9/28 T-wave acutely inverted Lead I Small complexes Consistent	5/ 2/29 Infarction—anterior wall left ventricle Vessels—left descending occluded; right, sclerotic but patent
7	12/ 9/28	12/30/28 Small complexes Flat T-Waves Indeterminate	1/ 2/29 Infarction—anterior wall left ventricle Vessels—left occluded at bifurcation; right, no obstruction
8	None	3/29/28 Left axis deviation T-waves inverted Lead I S.T. interval depressed Leads II, III Suggests anterior	4/ 3/28 Infarction—anterior wall left ventricle Vessels—left circumflex occluded; right, sclerotic but patent
9	10/20/35	11/29/35 QRS complex, relatively small and slurred Lead IV, absent Q, upright T Axial indeterminate Lead IV consistent	12/ 1/35 Infarction—anterior wall left ventricle with rupture Vessels—left descending occluded; right, sclerosis marked patent
10	6/12/36	6/26/36 Diphasic T-waves Leads II, III Lead IV, absent Q-wave Axial indeterminate Lead IV Consistent	7/ 2/36 Infarction—apex left ventricle Vessels—left descending occluded; right, sclerosis marked occluded
11	2/ 8/35	2/14/35 S.T. interval elevated Lead I, depressed Lead III Prominent Q_1 Diphasic T-wave Lead I Lead IV, S.T. interval depressed, small Q-wave. Axial consistent Lead IV consistent	4/15/35 Infarction—anterior wall left ventricle Vessels—left occluded

TABLE IV—CONT'D

CASE NO.	PAIN	EKG	AUTOPSY
12	12/24/34	12/27/34 S.T. interval elevated Lead I, depressed II, III. Slurring of S-wave all leads Flat T-wave Lead I Lead IV, absent Q, upright T Axial consistent Lead IV consistent	1/18/35 Infarction—anterior wall left ventricle and septum Vessels—left descending occluded; right, sclerotic but patent
13	8/23/36	8/24/36 S.T. interval depressed, Leads I, II Prominent Q_3 Lead IV, upright T-wave Axial suggests posterior Lead IV suggests anterior	8/27/36 Infarction—apex left ventricle Vessels—left occluded; right, sclerotic but patent
14	None	5/19/28 QRS complex, relatively small and slightly slurred Prominent Q_2 and Q_3 Axial consistent	5/23/28 Infarction—wall right ventricle Vessels—left no striking changes; right, occluded
15	10/25/31	11/ 7/31 S.T. interval depressed Lead I, elevated in II, III Prominent Q_2 and Q_3 Acutely inverted T_2 and T_3 Axial consistent	11/ 9/31 Infarction—left ventricle Vessels—left few plaques patent; right, occluded
16	None	12/10/30 Auricular flutter Indeterminate	12/15/30 Infarction—wall right ventricle Vessels—right occluded

dicative of the location or even of the presence of myocardial infarction Lead IV was of distinct help. Even with the additional precordial lead, the occasional case will not be entirely clear.

SUMMARY

This paper attempts to correlate present knowledge concerning electrocardiography with the diagnosis, prognosis, and localization of myocardial infarction. Several factors are worthy of mention. In the diagnosis of proved myocardial infarction the standard three lead electrocardiograms may accurately localize the infarction, may suggest infarction without accurate localization, may falsely suggest the area involved, or may show no change whatever. The addition of progress readings and of precordial leads will clarify many of these cases. In this series where the information is based chiefly on the axial leads only, posterior infarction carries practically as high an immediate and subsequent mortality as does the anterior. The careful work of Saphir and his associates points to the need for more careful and detailed analysis of both the myocardium and the coronary aborization at autopsy. Wider recognition of this need may well explain some of the inconsistencies between the electrocardiograms and the autopsy protocols indicated in this paper.

CONCLUSIONS

1. A review of 83 expired cases of coronary artery thrombosis which were studied electrocardiographically does not indicate any material prognostic

difference between Q_1T_1, or anterior type of electrocardiogram and the Q_3T_3 or posterior type.

2. The use of the standard three leads in coronary artery thrombosis without the addition of precordial leads has proved unsatisfactory in both the diagnosis and localization of myocardial infarction.

3. The addition of precordial leads gave definite indication of anterior myocardial infarction when the standard three leads alone did not, and in the cases where precordial leads were used there were no contradictory findings at autopsy.

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THE CHEMISTRY OF THE URINE AFTER RENAL DECAPSULATION FOR ANURIA*

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WE OBSERVED a case of anuria (five days of oliguria and two days of complete anuria) with complete recovery after decapsulation of one kidney. During this period the other kidney remained anuric, its function returning two weeks later. The entire output of urine was collected via the nephrostomy tube for a period of ten days. Further exact collections of urine were impossible because of removal of the nephrostomy tube. It was our intention to study the chemical composition of the urine over the entire ten-day period in order to ascertain the rapidity of excretion of the various chemical components after renal inactivity, and thus to obtain a picture of the degree of renal recovery.

T. F., No. A 1561, white female, aged 40 years, was admitted to the Mount Sinai Hospital on Dec. 2, 1937, and discharged Jan. 9, 1938.

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The patient was admitted for operative cystoscopy. Intravenous urography revealed a small calculus in the left ureter, about 5 cm. above the vesical orifice. Both kidneys excreted the dye in normal concentration. The left urinary tract was notably dilated above the stone.

At cystoscopy under nitrous oxide anesthesia the tight orifice of the left ureter was opened with electrical knife. Some bleeding was noted, but not more than usual. The patient left the operating room in good condition. The patient was voiding 2 to 3 ounces of slightly bloody urine every half hour. About four hours later the patient had a vomiting spell and became pulseless. No blood pressure could be obtained. Intravenous fluids were administered,

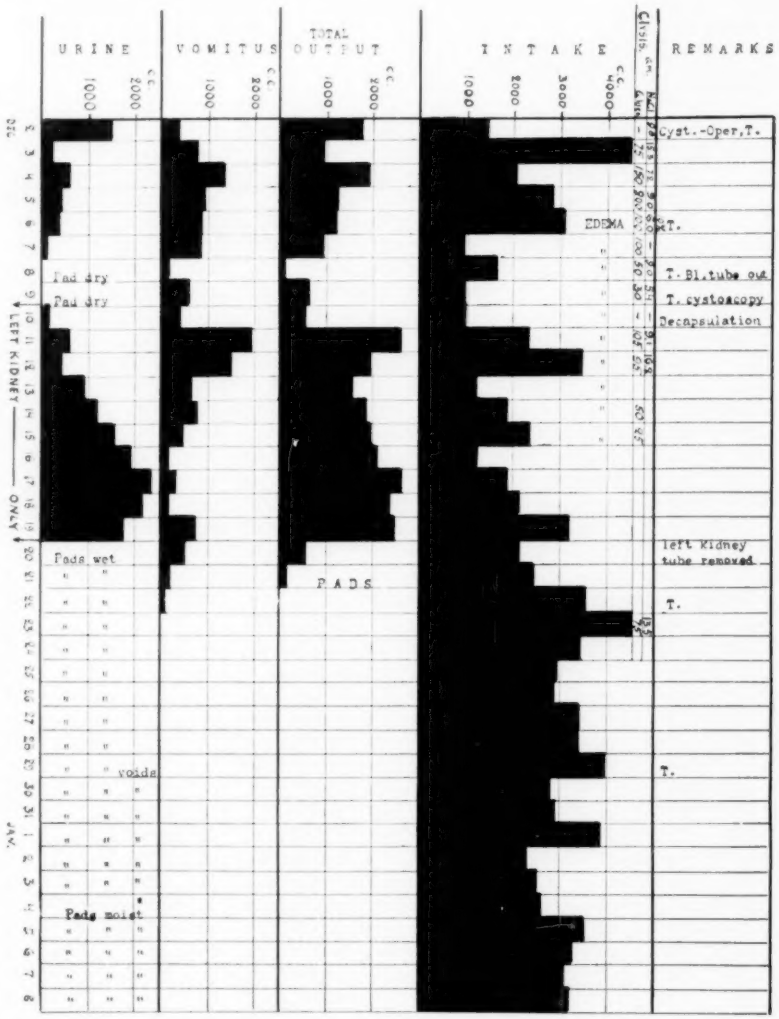

Fig. 1.—Master chart showing intake, total output, vomitus and urine, and the total intake of sugar and chlorides.

and the blood pressure returned to normal. On examination the bladder was found tremendously distended. Through a cystotomy incision about a quart of coagulated blood was evacuated from the bladder; the bleeding was coming from a small lively spurting artery at the mouth of the left ureter. Hemorrhage was stopped by suturing, and the wound was closed around a mushroom tube.

In spite of parenteral fluid administration vomiting persisted and the output continued very low (see Fig. 1) for the next five days, and finally complete anuria for two days. Left

renal decapsulation was performed on Dec. 10, 1937. The kidney was much enlarged and very hard. The kidney tissue bulged out markedly when the capsule was opened. The tissue was pale brown, evidence of low blood circulation. Following this procedure, the urine began flowing, mounting rapidly with the gradual complete disappearance of all symptoms.

COMMENT

These studies show some interesting facts (Fig. 2). The urinary output is gradual but slow in mounting in spite of the excess of fluids in the body. The specific gravity shows concentration with gradual dilution, the pH being slightly alkaline for the first two days, down to 5.6 during the next two days, back to

TABLE I

CHEMISTRY OF THE BLOOD BEFORE AND AFTER DECAPSULATION

	BEFORE	AFTER		
	12/9	12/11	12/13	12/22
B.U.N. (mg.)	100		62	115
Creatinine (mg.)	7.8			6.8
Uric acid (mg.)	8.0			
Carbon dioxide (vol. per cent)	56			49
Sugar (mg.)	185			
Chlorine (mg.)	420	380	385	550
Calcium (mg.)	8.2			
Total protein (gm.)	5.6			
Serum albumin (gm.)	4.3			
Serum globulin (gm.)	1.3			

neutrality and low alkalinity where it stayed until the ninth day, when it dropped back to the normal acidity. There was no sugar in any of the specimens in spite of a great excess of sugar in the tissues. Urea showed the usual slow excretion and was still low on the tenth day. The blood urea nitrogen was still 115 on the tenth day. Creatinine was excreted slowly, but much more

TABLE II

GROUPING OF URINARY COMPONENTS INTO THRESHOLD CLASSES AND PERCENTAGE OF THEIR EXCRETION AS COMPARED WITH NORMAL

		EXCRETION IN 24 HOURS			
		NORMAL	MAXIMUM	PER CENT	UNIT
High threshold	Glucose	0	0	0	
	Calcium	200	85.3	42.6	mg.
	Chloride	12	3.4	28.1	gm.
	Sodium	2.9	2.3	100.0	gm.
Low threshold	Urea	30.0	10.97	36.0	gm.
	Uric acid	700.0	726.8	100.0	mg.
	Phosphorus	2.5	3.34	100.0	gm.
No threshold	Creatinine	1200.0	1097.5	100.0	mg.
	Sulfur	3.0	3.3	100.0	gm.

rapidly than the urea, and reached normal on the tenth day. Uric acid behaved in the same manner as creatinine. Phosphorus, calcium, and chloride excretion were parallel and gradually mounted.

The loss of chlorides by constant vomiting before operation and for six days postoperatively was apparently not adequately balanced in spite of the saline clyses and hypertonic sodium chloride.

The present conception of the formation of urine is that the urine results from the glomerular filtration into Bowman's capsule of all the elements of the blood serum, except its protein content, and from the selective reabsorption of water and certain of the filtered substances by the tubules (Richards). Rehberg divides the soluble urinary constituents into three classes:

(1) Substances actively reabsorbed. These are conserved by the body and are present in lower concentration in the urine and in higher concentration in

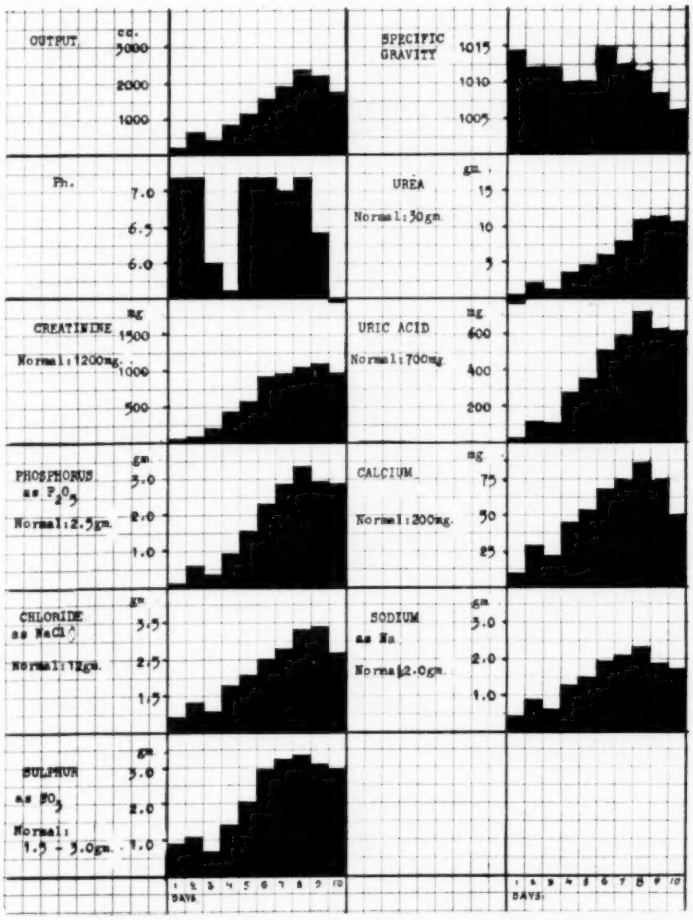

Fig. 2.—The excretion of the urinary components per twenty-four hours during the ten-day period.

the reabsorbed fluid than in the plasma. These are the so-called "high threshold substances," among which are included glucose, calcium, chloride, and sodium.

(2) Substances which diffuse through the tubular epithelium when the concentration in the tubular fluid is greater than in plasma. These are never actively reabsorbed. They are never found in lower concentration in the urine or in higher concentration in the reabsorbed fluid than in plasma. These are the so-called "low threshold substances," among which are urea, uric acid, and phosphates.

(3) Substances which are neither reabsorbed nor back diffused, the so-called "non-threshold substances," like creatinine and sulfates.

We have included in our analysis of the urine collected in the postdecapsulation period, representatives from these three classes of threshold substances. In general, it appears that at the end of the ten-day period, at which our study in its planned form had to be discontinued, the kidney had essentially recovered its functional integrity. At this period the kidney was excreting normal average quantities of at least one representative of each of the three classes listed above. The exceptions, calcium, chlorides, and urea, are not difficult to explain. The low calcium and chloride quantities may represent successful conservation of these substances by the body, since at this time the intake of these elements was below average in quantity. The patient was still losing chlorides by vomiting. The low urea output is not difficult to explain. The urinary output was still too low to have washed out the entire amount of urea accumulated in the blood since urea requires large amounts of water for complete elimination.

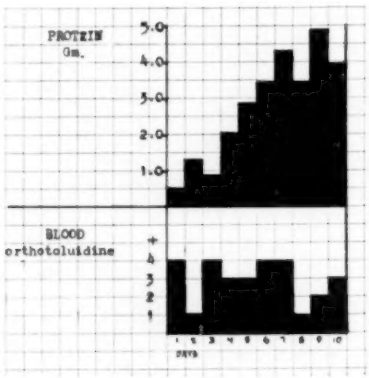

Fig. 3.—Parallel determination of blood and protein in the urine during the ten-day period to ascertain their quantitative relationship. It shows the amount of protein to be greater and out of proportion with the amount of blood, indicating true and heavy albuminuria.

It would appear that tubular function recovered very quickly since even on the first day no glucose appeared in the urine, and that the output was limited and conditioned chiefly by glomerular function, the latter limited, perhaps, in activity by the gradual reestablishment of adequate glomerular circulation. Thus, even at the end of the ten-day period, uncorrelated blood cells and albumin were present in the urine (Fig. 3).

Much of our findings can be adequately explained on the supposition that glomerular function was only gradually and imperfectly restored, while tubular function was quickly and satisfactorily regained.

There is no question that the decapsulation and that this procedure alone has restored the flow of urine from the kidney since the other kidney, remaining untouched, did not begin functioning spontaneously until two weeks later.

A pertinent question presents itself. Did the inactivity during the anuric state cause any alteration in either the glomerular or tubular function, or was the kidney stopped in its function just like stopping a clock and restarted without ill effect? Glomerular alteration should have evidenced itself by the presence

of albumin in the urine after decapsulation. Albumin was found in every specimen. Tubular injury would have evidenced itself by the presence of sugar in the urine. We found no evidence of sugar postoperatively.

It is apparent, therefore, that only one vital mechanism of the kidney was injured, namely, the glomerulus, while the tubular function was not altered.

One is unable to explain the cause of the anuria in this instance. We can only surmise that the glomerular function ceased because of tremendous swelling of the kidney, gradually increasing the tension within the renal capsule until the blood flow to the glomerulus ceased. This is supported by the observation on the operating table when we found the kidney pale brown and not red, evidence of great reduction in the quantity of circulating blood.

The blood pressure was either not high enough to overcome the greatly increased intrarenal pressure on the other side of the glomerular filter, or if it was sufficient and did permit normal filtration to proceed, the filtrate could not enter the compressed tubuli and further filtration ceased.

From all the data obtained, we must conclude that the anuria in this case was due to an idiopathic intrarenal edema. The edema was gradual in growth, as seen from the gradual decline of the urinary output, until the pressure became so great that by complete compression of the afferent blood supply to the glomeruli total anuria ensued. In order to determine the modus operandi of the vascular mechanism in anuria, several questions must be answered in the individual case of anuria: (1) Does the renal artery pulsate? (2) Does the renal cortex bleed? (3) Is there blood leaving the renal veins?

We are unable to answer these questions in this particular case as we did not think of them during the rapid operative procedure of the decapsulation. But these questions should be in the mind of every surgeon who is confronted with a case of anuria. Only by answering them correctly in many instances will the problem of true renal anuria be solved. The palpation of the renal artery is simple and quick in execution. The bleeding test of the cortex should not consume more than a few seconds. By making a few small superficial stab incisions, one can convince himself whether the cortex is rich or poor in blood. The observation on the renal vein is more difficult. One has to visualize the vein and by milking it, determine whether or not there exists an active return blood flow from the kidney.

By accumulating such data, the problem of true renal anuria will narrow itself down to a few causative agents and will thus enhance its future prevention and therapy.

CLINICAL OBSERVATIONS ON THE VALUE OF VARIOUS XANTHINE DERIVATIVES IN ANGINA PECTORIS*

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Askanazy[1] in 1895 was the first to report beneficial results with xanthines in angina pectoris. Since then a number of similar reports have appeared.[2-7] Evans and Hoyle[8] found that the xanthines did not compare in efficiency with glyceryl trinitrate in patients with angina pectoris. Riseman and Brown[7] concluded that the patients' reaction to various xanthines was too unreliable to judge their efficacy and to distinguish their comparative value. Placebos were found to give about the same degree of subjective relief as the various xanthines. They proposed to check the benefits objectively, using an exercise tolerance test leading to anginal pain as a measure of the results. With this objective method they reported benefits with xanthines in some patients, but the results with these drugs were not comparable to glyceryl trinitrate. Unfortunately, in our hands, the exercise tolerance test has not worked satisfactorily in that we[9] have not obtained the frequency of positive tests in patients with angina pectoris reported by Riseman and Brown.[7] We have therefore resorted to the patients' comparative subjective reactions to the various xanthines, placebos, and barbiturates when several of these were used in the same patients in succession.

PLAN OF STUDY

Fourteen patients were selected who were known to have angina pectoris, based on organic heart disease, for at least a year before starting the therapy. They were regular visitors in the Adult Cardiac Clinic in the Mandel Clinic and were able and willing to cooperate in this study by coming regularly and taking medication as prescribed. This study was carried on over a period of sixteen months beginning in June, 1936. Each of the 14 patients was placed on from three to fourteen courses of treatment, each course consisting of three weeks' medication of a single drug or a combination of drugs. The medication was taken in tablets four times daily, the tablets being given the patient directly by the investigator in such a way that the patient did not know the nature of the drugs given. No attempt was made to alter the handling of the patient in any other regard.

RESULTS

Table I summarizes the various courses of medication in the different patients classified according to the patient's report of the effect of the drug. It will be seen that they fall into four courses: xanthines (A, B, C), pheno-

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barbital (F, H), placebos (D, E), and combinations of xanthines with phenobarbital (I, J, K, L).

TABLE I

EFFECT OF DRUGS ON ANGINA PECTORIS

PATIENT	SATISFACTORY RELIEF OBTAINED WITH THE FOLLOWING MEDICATION	MILD RELIEF OBTAINED WITH THE FOLLOWING MEDICATION	NO RELIEF OBTAINED WITH THE FOLLOWING MEDICATION
1	A, A, L, L	B	B, E, K
2	B, F		A, B, B, C, D, D, E
3	A		C, E, F, L
4	C, I, I, I, I, J, K, K, L	A	B, C, C, H
5	A, A, A		E
6	A, B, C, J, K	B, D, D, K, L	B, E, F, I
7	C, C, D, E		B, B, D, F
8	I, I, I		K
9	A		A, A
10	J, L		H
11	I, I, I, K, L		H
12	F		J, K, L
13	H, I, I, K		A, E, F, F, H, I
14	I, I, I		F

A. Theocalcin, theobromine calcium salicylate, gr. vii ss. q.i.d., Bilhuber-Knoll & Co.
B. Phyllicin (theophyllin calcium salicylate), gr. v q.i.d., Bilhuber-Knoll & Co.
C. Aminophyllin (theophyllin ethylenediamine), gr. i ss. q.i.d., Dubin Laboratories.
D. Acetyl salicylic acid, gr. v q.i.d.
E. Sodium bicarbonate, gr. x q.i.d.
F. Phenobarbital, gr. ss. q.i.d.
H. E. plus F.
I. Theobromine sodium salicylate gr. v with phenobarbital gr. ss. q.i.d.
J. C. plus F.
K. B. plus F.
L. A. plus F.

Table II shows the results with the xanthines, Table III the results obtained with placebos and phenobarbital, and Table IV the results obtained with combinations of xanthine and phenobarbital.

TABLE II

EFFECT OF XANTHINES ALONE ON ANGINA PECTORIS

DRUG COMBINATION	PATIENTS	COURSES	COURSES GIVING RELIEF	COURSES GIVING NO RELIEF
A*	8	13	9 (69 per cent)	4 (31 per cent)
B*	5	12	5 (42 per cent)	7 (58 per cent)
C*	5	8	4 (50 per cent)	4 (50 per cent)
Total		33	18 (55 per cent)	15 (45 per cent)

*See Table I footnote.

TABLE III

EFFECT OF PLACEBOS AND PHENOBARBITAL ON ANGINA PECTORIS

DRUG COMBINATION	PATIENTS	COURSES	COURSES GIVING RELIEF	COURSES GIVING NO RELIEF
D*	3	5	2 (40 per cent)	3 (60 per cent)
E*	7	8	1 (13 per cent)	7 (87 per cent)
F*	6	7	2 (29 per cent)	5 (71 per cent)
H*	4	5	1 (20 per cent)	4 (80 per cent)
Total		25	6 (24 per cent)	19 (76 per cent)

*See Table I footnote.

TABLE IV

EFFECT OF COMBINATION OF XANTHINES WITH PHENOBARBITAL ON ANGINA PECTORIS

DRUG COMBINATION	PATIENTS	COURSES	COURSES GIVING RELIEF	COURSES GIVING NO RELIEF
I*	6	17	15 (88 per cent)	2 (12 per cent)
J*	4	4	3 (75 per cent)	1 (25 per cent)
K*	7	9	6 (67 per cent)	3 (33 per cent)
L	7	8	6 (75 per cent)	2 (25 per cent)
Total		38	30 (79 per cent)	8 (21 per cent)

*See Table I footnote.

The following were the criteria used in establishing the benefits of medication: Satisfactory relief with one or more drugs was considered present when the patient reported that he was able to do his usual day's work with little or no precordial pain, and felt that the medicine he was taking was giving him satisfactory relief, so that he used no nitroglycerin except on rare occasions. Mild relief with the medication was considered present when the patient volunteered the information that he received relief at times and had reduced his nitroglycerin medication considerably. No relief with a drug combination was considered present when the patient noted very little or no relief and reported the need of nitroglycerin in the usual amounts.

On this basis it was found that phenobarbital, sodium bicarbonate, and their combination, as well as aspirin, gave relief in about one-fourth of the courses. The xanthines gave relief in one-half the courses, while the combination of xanthine with phenobarbital gave relief in four-fifths of the courses. Theocalcin alone is an exception to the other xanthine derivatives used in that relief was obtained in seven-tenths of the courses.

SUMMARY

These results are significant in indicating that:

(1) The xanthines used, especially theocalcin, offer a better chance for relief of angina pectoris, in the ratio of 2 to 1, than aspirin, sodium bicarbonate, phenobarbital, or a combination of the latter two.

(2) The combination of a xanthine with phenobarbital offers a much better chance for relief of angina pectoris, in the ratio of more than 3 to 1, than the phenobarbital alone or in combination with sodium bicarbonate.

(3) The combination of a xanthine with phenobarbital offers a better chance for relief of angina, in the ratio 1.5 to 1, than xanthine alone.

(4) While the errors inherent in such types of studies as these are large and the series of courses of therapy used are relatively small, the results, we believe, cannot be dismissed as without significance.

(5) It is, therefore, concluded that xanthine derivatives do have a beneficial effect on the subjective state of the patient with angina pectoris and that this effect is definitely enhanced when the xanthine is combined with a sedative like phenobarbital.

425 S. PULASKI RD.

I wish to acknowledge my indebtedness to Dr. Louis N. Katz at whose suggestion this study was undertaken, for his guidance in the conduct of the investigation and for his advice in preparing this report. I am indebted to Dr. W. C. Buchbinder for his advice in the selection of patients for this study.

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THE EFFECTS OF BACTERIAL TOXINS ON ERYTHROGENESIS*

A Study of the Peripheral Blood Picture in Rabbits Made Anemic by Chronic Hemorrhage

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IN THE normal individual a remarkably constant balance between the forces of blood destruction and blood regeneration is maintained. This physiologic equilibrium is subject to the influence of every disease process in the body, local or systemic, by reason of the special functions of the blood and its distribution to all parts of the body. Of the significant disturbances the most important general group is that of the anemias. On the basis of recent advances in hematology, and by the correlation of clinical and laboratory studies, Haden[1] has suggested the following classification of the anemias: (1) anemia due to acute hemorrhage; (2) anemia caused by excessive blood destruction; (3) anemia resulting from depression of bone marrow function; and (4) anemia due to the absence of specific substances necessary in the building of normal erythrocytes. Often a combination of these factors is at work in the production of an anemia. In the first group mentioned the cause of the anemia is obvious. The fourth general group includes those anemias which result from specific deficiencies in the building materials and catalyst-like substances which are necessary in the formation and release of normal erythrocytes to the peripheral circulation. The results of deficiencies in two such elements, namely, iron and antianemic substance, are definitely recognized. Sabin[2] has shown the points of action of these two substances in the maturation of the normal red blood cell. The great majority of the anemias, however, are included in groups 2 and 3. Anemias resulting from excessive erythrocyte destruction within the body may

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be due to hemolytic poisons such as mushrooms, nitrobenzol, and snake venom, to bacterial toxins, to destruction of red cells by malarial and other parasites, or to abnormal erythrocyte fragility. Marrow poisons, such as benzol and lead, bacterial toxins, excessive irradiation of the bone marrow, and invasion and replacement or destruction of marrow by other tissue (osteosclerosis, malignancy, leucemia, etc.) are recognized as causes for the anemias due to depressed bone marrow activity.

It is significant that bacterial toxins appear as causative factors in both groups 2 and 3. Infections are unquestionably the greatest single cause of anemia and they produce the greatest variety of clinical types. So variable are the results of clinical and laboratory studies within this group that they possess no group diagnostic value. The mechanism by which anemic states are produced by bacteria and their toxins has had no adequate explanation. Present evidence would seem to indicate that there are probably a concomitant depression of the bone marrow and an increased red cell destruction.[3]

Further studies on the etiologic relationship of bacterial toxins to the anemias would be valuable. The present investigation was made with this thought in mind. In this series of experiments the data have been obtained by study of the peripheral blood picture. Investigations by Steele[4] and others[5, 6] have shown the close correlation of the bone marrow pattern and the peripheral blood picture. The reticulocyte count is a sensitive and reliable index to the state of erythrogenic activity of the bone marrow. Steele said, "The marrow finds direct expression in terms of the cells of the blood." For this investigation standard tetanus and diphtheria toxins, streptococcus and staphylococcus hemotoxins, and diphtheria toxoid were used.

METHOD

These studies were made on adult rabbits taken from stock. Any rabbit showing signs of disease, injury, or other abnormal change was discarded and its records were excluded from the general data. An adequate diet was given to the rabbits and water was available at all times.

Each rabbit was observed for a varying period to determine normal blood values. Red and white blood cell counts, hemoglobin values, reticulocyte counts, and rectal temperatures were taken at frequent intervals. The normal control period was followed by a period during which the rabbits were subjected to frequent bleedings, so regulated as to produce a chronic anemia with as constant a reticulocytosis as possible.

The rabbits were made anemic by repeated bleedings to stimulate reticulocyte production. In the presence of increased erythrogenic activity, variations in the degree of reticulocyte response are shown more strikingly and minor changes are more clearly discernible. Also, chronic hemorrhage creates a constant demand for red blood cells in the peripheral circulation. Erythrocytes are not stored, but are rapidly delivered to the peripheral blood, and thus a possible source of error is eliminated.

While in this chronic anemic state, the rabbits were given the various bacterial toxins. Subsequent red and white blood cell and reticulocyte counts and hemoglobin values were taken until the animal died or until significant blood changes had ceased. Concomitant records of weights and temperatures were kept.

Blood was drawn by puncture of a vein at the base of the ear. Standard pipettes and counting chambers were used in making the red and white cell counts. Hemoglobin levels were determined by the Newcomer method; on the instrument used, 100 per cent was equivalent to 15.6 gm. of hemoglobin in each 100 c.c. of blood. Reticulocyte counts were made from blood smears prepared on cover slips stained with brilliant cresyl blue and counterstained with Wright's stain. These smears were also examined for variations in size and shape of the red blood cells and for the presence of normoblasts.

In administering the various toxins and bacterial products to the rabbits no standard procedure was followed. With the exception of the *Staphylococcus aureus* hemotoxin all injections were given subcutaneously; this product was injected into the muscles of the thigh. The dosages of the toxins used were varied. Doses of tetanus toxin, ranging from sublethal amounts up to several times the lethal dose, were given. By a process of trial and error the lethal dose of diphtheria toxin was determined and sublethal and finally lethal doses were given. Increasingly large doses of streptococcus and staphylococcus hemotoxins and of diphtheria toxoid were administered.

Necropsy was performed on all animals that died. Portions of tibial bone marrow, spleen, liver, and kidney were fixed in Zenker's and Carnot's solutions. The tissue was sectioned and stained with hematoxylin-eosin and Giemsa stains.

THE EFFECTS OF TETANUS TOXIN

The rabbit possesses considerable natural resistance to tetanus toxin. When this toxin is injected subcutaneously, the subsequent developments depend upon the rate of administration as well as the total amount of toxin given. A single lethal dose will cause death in seven or eight days in the average rabbit. Five or six days after the injection of the toxin, muscle spasms develop near the site of the injection and gradually spread to the rest of the body. The muscle hypertonicity increases and convulsions occur after stimulation, followed by intervals during which the animal lies exhausted. Finally the animal dies in a state of extreme general tonic contraction. If the first or first few injections are not sufficient to overcome the rabbit's natural resistance and its rapidly developing acquired immunity, it does not succumb. When sublethal doses are employed in the beginning, the amount of toxin can be rapidly increased to many times the lethal dose without causing permanent damage to the rabbit.

The peripheral blood values of six rabbits were determined by frequent examinations for a period of several weeks and the animals then subjected to repeated bleedings. After the bleedings were begun, there was an immediate reticulocyte response in each case. The rapidity with which the reticulocyte percentage rose depended upon the degree of hemorrhage to which the animals were subjected and to individual differences in response. In general, the more quickly blood was taken, the quicker the response. For a few days the absolute number of reticulocytes per cubic millimeter was usually relatively less than the reticulocyte percentage because of the loss of many red blood cells. Early observations indicated that if the frequency and size of the bleedings were so regulated as to hold the red blood cell count between 3,500,000 and 4,000,000 per c.mm., there would be established a fairly constant reticulocytosis, averaging about 20 per cent and equivalent to an absolute number of reticulocytes ranging

from 600,000 to 800,000 per c.mm. This anemic state could be maintained for months and reticulocytosis held at a high level. The anemic control period was considered that period between the first high point in the reticulocyte level and the beginning of toxin administration. Standard tetanus toxin was administered subcutaneously to the animals in varying doses and at various intervals. Blood studies were continued until the animals died or until significant changes in the peripheral picture had ceased.

The most striking observation was the marked contrast in the values obtained from those animals which had received a lethal dose of the toxin and those which had not. In the former group, during the period of intoxication a very dramatic drop occurred in reticulocyte production, whereas in the latter group this was slight and transient.

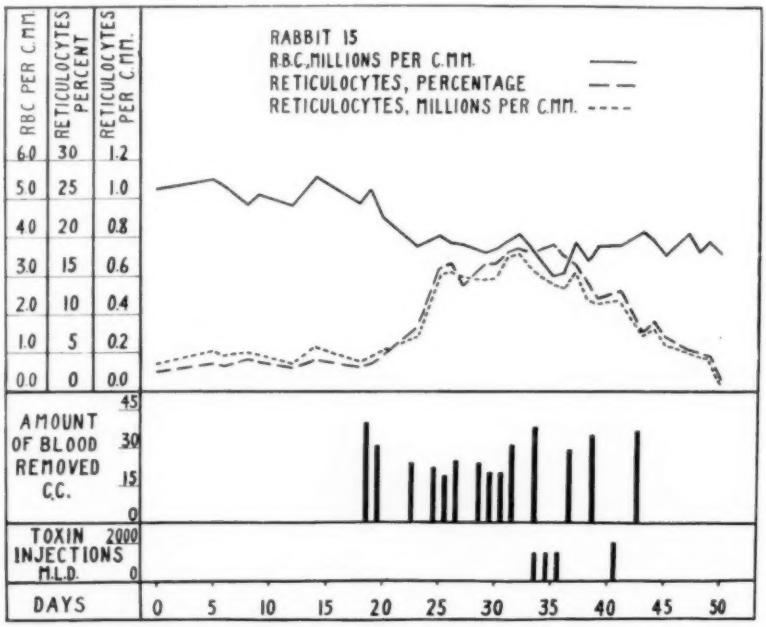

Chart 1.—Observations on the blood of Rabbit 15 injected with tetanus toxin in lethal amount.

During the anemic control period the average red blood cell count for animals receiving a lethal dose of the toxin was 1,760,000 per c.mm. lower than in the normal control period. The average reticulocyte count was 670,000 per c.mm., or almost five times greater than the normal value. The average daily bleeding during the anemic control period was 20.3 c.c. From the third to the seventh day following the administration of toxin a decline began in the reticulocyte percentage which was progressive until death occurred. The average red cell count of 3,410,000 per c.mm. at the time of the original injection of toxin corresponded closely to that at the termination of the period, 3,610,000 per c.mm., whereas the absolute number of reticulocytes had dropped off from an original 720,000 per c.mm. to a terminal 140,000, or more than a five-fold reduction. After the toxin injections were begun, rabbits receiving lethal amounts of the toxin were bled less often, and much less blood was removed than during

the anemic control period. During the period of intoxication the average daily bleeding was only 10.8 c.c. as compared to a daily average of 20.3 c.c. during the anemic control period. By so reducing the size and frequency of the bleedings following the toxin injections, the red cell count was maintained at the pretoxin level, but there was a marked, progressive decline in reticulocyte values. Chart 1 illustrates in graphic form the course of blood values in Rabbit 15, which is typical for the animals receiving a lethal dose of toxin.

The data for animals receiving sublethal doses of the toxin were very similar to those in the group above up to the point at which toxin administration was begun. Subsequently the two groups were in marked contrast. In the animals receiving sublethal intoxications only transient changes were noted in the periph-

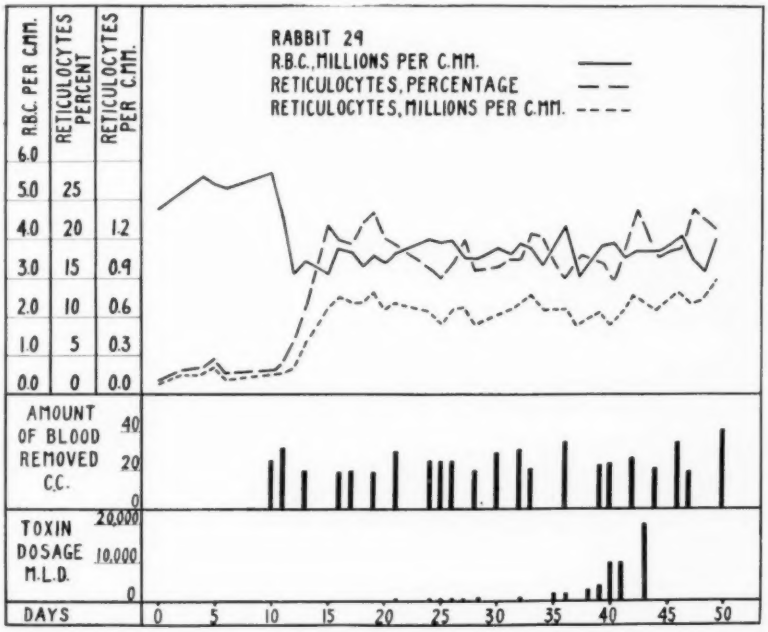

Chart 2.—Observations on the blood of Rabbit 29 injected with tetanus toxin so as to produce sublethal intoxication.

eral blood. After a short reticulocyte depression of varying degree, reticulocytes again appeared in numbers equal to those present during the anemic control period. The average daily bleeding after toxin injection was 14.5 c.c. as compared to 16.8 c.c. for the anemic control period. Terminal red blood cell and hemoglobin values varied only slightly from pretoxin levels. In Chart 2 are presented blood values of Rabbit 29, which are typical for the animals receiving sublethal doses of the toxin.

In the early stage of the anemia in all rabbits numerous large reticulocytes, heavily laden with hemoglobin, were seen in the blood smears. After the anemia had existed for some time, occasional normoblasts were always present. Polychromatophilia and anisocytosis were constantly noted. The average final red cell count for all animals was 66 per cent of the control value, whereas the final hemoglobin level was 70 per cent of the normal value. Thus, in spite of long-continued bleedings, the bone marrow delivered normal or slightly hyper-

chromic red blood cells into the peripheral circulation. In some instances there was a slight temperature rise following the toxin injections, but these changes were not constant. When the intoxication was lethal, a subnormal temperature existed for some time before death. Without exception the animals gained in weight through the normal and anemic control periods. Those that died lost from 1½ to 3 pounds in the terminal stages. Those not receiving a lethal dose showed no characteristic or significant changes in weight. No constant variations in the white blood cell counts were noted.

At post-mortem examination the red marrow of the animals that died was definitely hyperplastic. Red marrow filled the cavity of the femur and occupied two-thirds or more of the proximal end of the tibia. Microscopic examination

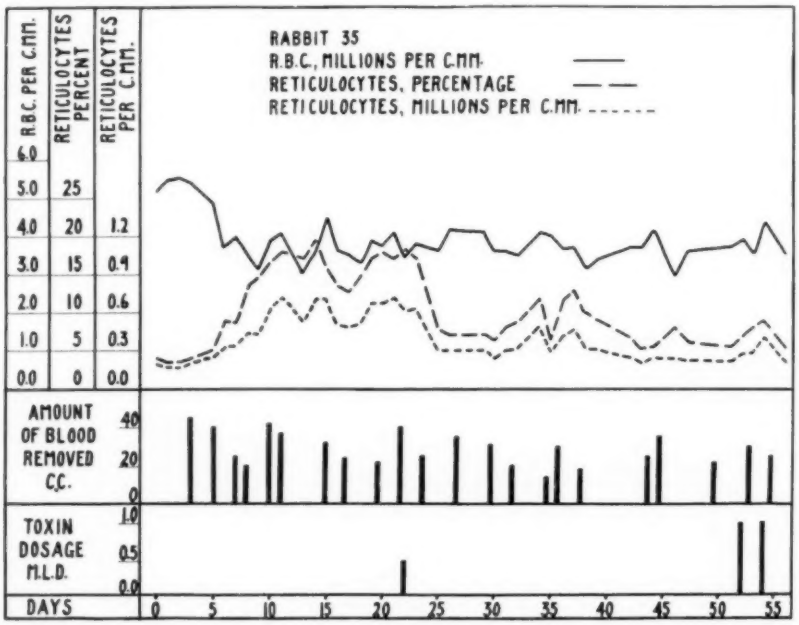

Chart 3.—Observations on the blood of Rabbit 35 injected with diphtheria toxin.

of certain sections of the bone marrow showed a highly cellular marrow and a decrease in fat content. The sinusoids were dilated and engorged, and there appeared to be an increase in the proportion of erythroid cells. There was relatively little nuclear division. Other marrow sections, probably from the lower third of the tibia, were not hyperplastic, indicating that there was still room for expansion of the hematopoietic function. Sections of the spleen did not show an unusual amount of phagocytosis of red blood cells or of nuclear debris. Some showed a moderate amount of material having the appearance of hemosiderin, but this was not particularly striking. Sections of the liver showed areas of toxic degeneration.

EFFECTS OF DIPHTHERIA TOXIN AND TOXOID

A lethal dose of diphtheria toxin injected subcutaneously into a rabbit will cause death in eighteen to ninety-six hours, depending upon the size of the

dose. If a series of smaller doses is given so as to produce lethal intoxication, the animal will survive longer and show progressive paralysis and cachexia.

The peripheral blood of three rabbits was examined frequently for a period of several days to determine normal blood values. A fairly constant anemia was established by chronic hemorrhage. Two of the animals were given subcutaneous injections of varying doses of diphtheria toxin, and the third received repeated doses of diphtheria toxoid.

At the beginning of the period of intoxication reticulocyte values for both rabbits receiving diphtheria toxin were high, namely, an average of 690,000 per c.mm., as compared to the normal value of 180,000. By means of several trials the lethal dose of toxin for a rabbit was determined. To one of the rabbits a lethal dose was administered in a period of four days. Immediately after the

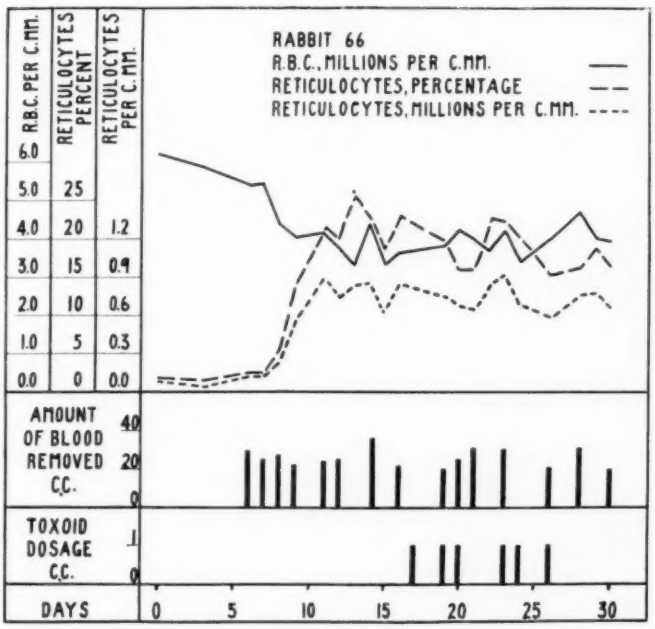

Chart 4.—Observations on the blood of Rabbit 66 injected with diphtheria toxoid.

first injection there began a rapid reduction in the number of reticulocytes in the peripheral blood which continued until death. To the other rabbit a single sublethal dose was first given and the blood values followed for one month before a subsequent injection of toxin. During this period of observation and while the red blood cell level was maintained fairly constantly, there was a drastic reduction in the number of reticulocytes which persisted. Further fatal doses of the toxin produced a still greater drop of reticulocytes. Values for Rabbit 35 are shown graphically in Chart 3.

Although in the one case the period of intoxication was short and in the other it was prolonged, the blood values show very similar general trends. Whereas the average pretoxin red blood cell count was 3,310,000 and the absolute number of reticulocytes 690,000 per c.mm., the terminal red blood cell count was 3,060,000 and the reticulocytes 210,000 per c.mm. This more than threefold reduction in the reticulocytes would have been greater had the terminal red blood

cell count coincided more closely with the former, for, in these prolonged chronic anemias, additional bleeding with reduction of the red blood cell count always caused an increased outpouring of reticulocytes in the peripheral blood. Compared to an average daily bleeding of 14.6 c.c. during the anemic control period, only 9.5 c.c. were taken each day during the period of intoxication. Thus, while one-third less blood was being taken daily and the red cell count decreased slightly, a very conspicuous reduction occurred in the number of reticulocytes in the peripheral blood.

Examination of the blood smears revealed frequent normoblasts. In the later stages anisocytosis was prominent, and many of the cells were extremely hypochromic. The terminal hemoglobin value was 52 per cent of the normal value, and the red blood cell count was 57 per cent of the normal; hence, toward the end of the course there was a drop in the color index. During the period of intoxication there was a gradual weight loss, varying from 1¼ to 2¾ pounds. Each toxin injection was followed by a temporary temperature rise up to as much as 1° F. The toxin also produced a varying degree of leucocytosis. At necropsy an extension of the red marrow of these animals was apparent and the marrow showed definite hyperplastic changes.

Chart 4 shows the values in the rabbit given diphtheria toxoid. This material was administered subcutaneously in 1 c.c. doses and was repeated every second to fourth day until the animal succumbed. The variations in the red blood cell and reticulocyte counts were no greater than might have been expected had the diphtheria toxoid not been given. The percentage and absolute number of reticulocytes remained high until the death of the animal. Death was attended by an extreme diarrhea, with rapid dehydration and wasting. A striking fact in this case, although the bleedings were not unusually excessive or prolonged, was the low hemoglobin level. Whereas the final red blood cell count was 69.8 per cent of the normal control value, the terminal hemoglobin value was only 52.8 per cent of the original. Whether the diphtheria toxoid acted directly or indirectly to produce this marked lowering of the color index is a conjectural point.

EFFECT OF STREPTOCOCCUS AND STAPHYLOCOCCUS HEMOTOXINS

The pathogenicity of the soluble exotoxins of *Streptococcus pyogenes* for rabbits varies greatly, depending on the virulence of the strain. For this study the toxin of *Streptococcus scarlatinae* was chosen as fairly characteristic of the toxins produced by this group of organisms. The staphylococcus toxin was prepared from a strain of *Staphylococcus aureus* isolated from a furuncle. It was ascertained that this strain possessed a fairly high toxigenic capacity and produced a filtrable toxin which was hemolytic. The exact unit strength of these toxins was not determined, but they were considered to be representative of the two groups of organisms.

The peripheral blood values of four rabbits were determined by frequent examinations for several days, and the animals were then subjected to repeated bleedings. When the reticulocyte response had reached a high sustained level, two of the rabbits were given increasingly large doses of *Streptococcus scarlatinae* hemotoxin subcutaneously. The other two received increasing intramuscular injections of *Staphylococcus aureus* hemotoxin.

Chart 5 shows graphically the course of peripheral blood values of Rabbit 72, which is typical for both rabbits receiving streptococcus toxin. The total dosage given each of the animals was very large. Early small doses produced a reduction in the number of reticulocytes which was only temporary; to maintain the lower reticulocyte values the dosage had to be rapidly increased. This fact is best illustrated by the course of Rabbit 72 in which the reticulocytes after a transient depression rose almost to the pretoxin level and only after very large doses of the toxin did they drop to a low level. The average final value of reticulocytes in the peripheral blood of both animals was 380,000 per c.mm. as compared to pretoxin value of 870,000; this drastic reduction occurred with scarcely any change in the red blood cell count or in the hemoglobin percentage.

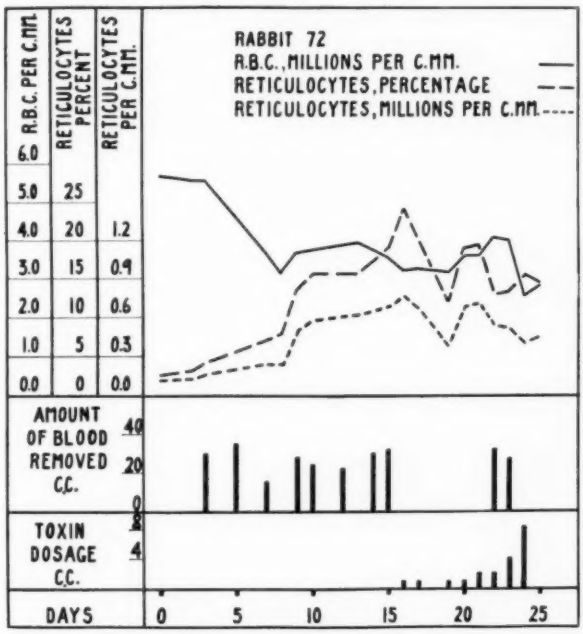

Chart 5.—Observations on the blood of Rabbit 72 injected with *Streptococcus scarlatinae* hemotoxin.

The average daily bleeding was only 7.6 c.c. during the period of intoxication as compared to 16.4 c.c. for the anemic control period.

The toxin injections occasionally caused a temporary rise in temperature (in one case, 2.5° F.), but this change was not constant. A small increase in the number of leucocytes followed the toxin administration. No significant weight changes were noted. Blood smears showed anisocytosis, polychromatophilia, and occasional normoblasts.

Chart 6 shows the course of blood values of Rabbit 68 which received staphylococcus toxin. For several days this animal was given relatively small doses of the toxin. After a rest period of ten days in which no toxin was given, injections of larger doses were resumed. The initial injections produced a decrease in the number of reticulocytes, but during the rest period it increased to the pretoxin level. When toxin administration was resumed, the reticulocytes again fell off rapidly. A second rabbit was given five increasingly large doses

of the toxin on consecutive days. In this case, there was a progressive reduction in the number of reticulocytes. The average reticulocyte count for both rabbits at the termination of the study was 360,000 as compared to 690,000 per c.mm. for the anemic control period and 530,000 just before the toxin administration. An average of 5.6 c.c. less blood was taken daily during the period of intoxication than during the anemic control period. The fact that as much blood was taken daily from Rabbit 68 after the beginning of toxin injections as before can be ascribed chiefly to the rest period of ten days between injections, when the reticulocytes reached the pretoxin level. Average values revealed that staphylococcus hemotoxin in large doses produced a definite reduction in the number of reticulocytes in the peripheral circulation.

Injections of this toxin called forth a moderate leucocytosis in both animals. Slight temperature elevations sometimes occurred following the toxin, but these

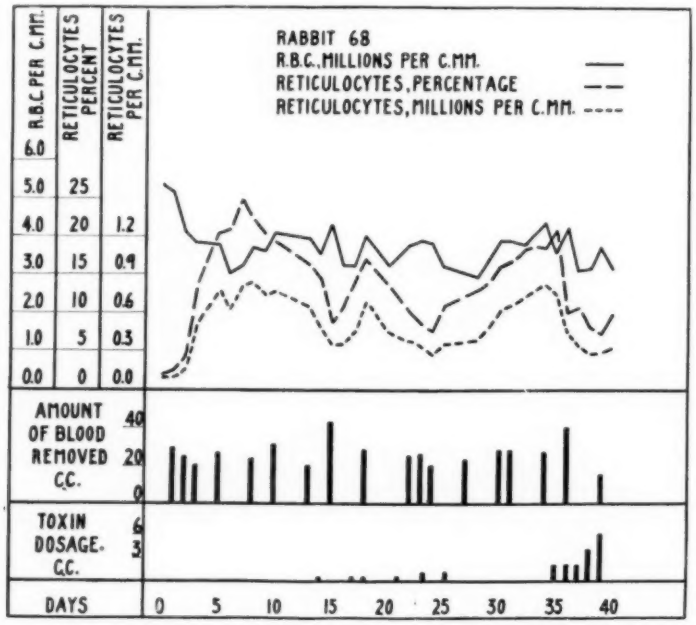

Chart 6.—Observations on the blood of Rabbit 68 injected with *Staphylococcus aureus* hemotoxin.

were transient and not constantly observed. Both animals lost a small amount of weight, ¼ to ½ pound, during the period of intoxication. Examination of the blood smears showed anisocytosis, polychromatophilia, and occasional normoblasts.

COMMENT

The mechanism by which bacterial toxins produce anemia has not been fully explained. In the course of acute bacterial infections, in which the blood stream is invaded, there is increased hemolysis of red blood cells. To an unknown extent such infections are believed also to exert a depressing action on bone marrow activity.[7] In the very common "secondary anemias" associated with chronic infections, the process by which the anemia is produced is more obscure. Yet in such chronic infections as rheumatic fever and chronic osteo-

myelitis there is often a profound anemia. At times small, hidden, or apparently unimportant foci of infection may be responsible for an anemia of moderate degree.

In this study additional data concerning the origin of this type of anemia have been collected by a study of the peripheral blood. In order that minor changes in the marrow activity might be discerned and all variations be brought out more strikingly, the marrow was thrown into a high degree of erythrogenic activity by repeated bleedings. Also, in the presence of a chronic anemia, when there is a constant demand for more red blood cells in the peripheral circulation, young cells are rapidly delivered to the blood. By maintaining this want for more cells, the storage of mature cells in the marrow or other organs is precluded, and thus a possible source of error is eliminated.

The administration of bacterial toxins to rabbits previously made anemic by bleeding produced a distinct reduction in reticulocyte values in all cases. In the intoxicated animals the average daily bleeding required to maintain the red blood cell count at the pretoxin level was reduced in every instance. It is obvious that the toxins entered into the picture as etiologic factors in the maintenance of the anemia. If the action of toxin had been primarily that of internal blood destruction, then reticulocytosis should have been further stimulated, just as additional bleeding adds an extra impetus to blood formation, but this was not true.

The red bone marrow of the animals that were examined at necropsy was definitely hyperplastic. Histologic examination showed the tissue to be highly cellular, with an apparent increase in the proportion of erythroid cells, and the sinusoids dilated and engorged. The absence of evidence of unusual erythrophagocytosis in the spleen sections suggested that erythrocytes were not being destroyed in excessive amounts. This seemed to indicate that the failure to maintain the red blood cells in the peripheral blood at the pretoxin level was due to factors relating to the bone marrow. Declining reticulocyte counts with associated reductions in the amount of daily bleeding in the presence of marrow hyperplasia suggested that the mechanism was an interference with the maturation of cells and their delivery by the bone marrow to the peripheral blood. Significant changes in the marrow activity, as revealed by a study of the peripheral blood, are shown in Table I.

TABLE I

AVERAGE RETICULOCYTE VALUES AND BLEEDING DATA OF RABBITS BEFORE AND AFTER THE INJECTION OF VARIOUS BACTERIAL TOXINS

BACTERIAL TOXIN USED	AVERAGE PRETOXIN RETICULOCYTE VALUE	AVERAGE TERMINAL RETICULOCYTE VALUE	AVERAGE DAILY BLEEDING ANEMIC CONTROL PERIOD	AVERAGE DAILY BLEEDING PERIOD OF INTOXICATION
	Millions per c.mm.	*Millions per c.mm.*	*c.c.*	*c.c.*
Tetanus toxin				
1. Lethal amount	0.72	0.14	20.3	10.8
2. Sublethal amount	0.72	0.73	16.8	14.5
Diphtheria toxin	0.69	0.21	14.6	9.5
Diphtheria toxoid	0.85	0.76	17.8	14.9
Streptococcus hemotoxin	0.87	0.38	16.4	7.6
Staphylococcus hemotoxin	0.53	0.36	13.4	7.8

Other factors have been shown to inhibit marrow activity. In a series of anemias of varied etiology, Riech[8] showed that large transfusions were followed by a drop in reticulocytes in the peripheral blood and interpreted this change as probably due to a decrease in the normal stimulus for marrow activity. Smithburn and Zerfas[9] have pointed out that during the period of hyperpyrexia in a bacterial infection there was a marked reduction in reticulocytosis. In a study of the anemia which occurs in cases of chronic nitrogen retention, Parsons and Ekola-Strolberg[10] concluded that the anemia was produced by depression of bone marrow activity. The effect of fever alone upon reticulocyte production and the effects of foreign protein shock and the phenomenon of hypersensitivity on marrow activity have not yet been explained.

SUMMARY

Representative bacterial toxins were administered to rabbits in which a high degree of erythrogenic activity had been produced by chronic hemorrhage. The red blood cell count and hemoglobin value were maintained at fairly constant levels by regulating the degree of hemorrhage.

Study of the peripheral blood revealed in all cases that bacterial intoxication produces a definite but variable reduction in reticulocyte values. Standard tetanus and diphtheria toxins in lethal doses markedly interfered with normal marrow activity. Sublethal doses produced temporary changes. Diphtheria toxoid caused little effect. Streptococcus and staphylococcus hemotoxins produced definite but milder interference with erythrogenesis.

In the period of intoxication, the average daily bleeding required to maintain the pretoxin anemic level was reduced in all cases. The absence of evidence of excessive red blood cell destruction pointed to disturbed bone marrow activity. Evidence presented here indicates that interference with normal marrow activity is an important factor in the production of anemias by bacterial toxins and suggests that the mechanism is an inhibition of maturation of the erythrocytes in the bone marrow, with consequent failure of delivery of adequate numbers of cells to the peripheral blood.

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AN ATTEMPT TO MOBILIZE LIPOIDS FROM STORAGE DEPOTS BY DEEP MASSAGE AND INCREASED TISSUE TEMPERATURES*

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THE object of this study was to determine whether or not fats could be mobilized from their depots by mechanical or thermal means. Heat and massage have been employed for reduction purposes from time immemorial. Claims for local removal of fat by massage are often made. If it be possible to mobilize fat by this means, would lipoids appear in the blood stream in sufficient quantities to be detectable by quantitative chemical analysis?

Body lipoids are usually classified under three headings: (a) true fats, or tryglycerides of fatty acids; (b) lipids, or phospholipids, combinations of fatty acids with phosphorus, either with or without nitrogen; (c) nonsaponifiable substances of somewhat similar nature, such as the sterols, of which cholesterol is the chief representative in the animal organism. Distinctions are sometimes made between the tissue fat and the storage or depot fat, for which there is some justification. Fats are readily mobilized from the storage depots in response to a demand for fuel. In extreme emaciation, however, a certain amount still remains in the tissues. This tissue fat has been called by the French the "élément constant" and has been found to consist of phospholipids. This does not mean that phospholipids are unavailable for heat and energy, but depot fat seems to be more readily utilizable for this purpose.

Human depot fat is composed largely of stearin, palmatin, and olein. The first two are fully saturated and are solid at body temperature. Olein is unsaturated and liquid at even less than body temperature. This mixture of depot fats is liquid in the body because of the high proportion of olein which comprises approximately 75 per cent of the mixture. Stearin in present only in a negligible amount, while palmatin is present to the extent of about 20 per cent.

Just how fats are mobilized to the tissues is somewhat obscure. In the intestines they are emulsified by the bile, and split by the lipase of the pancreatic secretion and the succus entericus. They are then absorbed into the lacteals and carried to the thoracic duct through which they obtain entrance to the blood stream. Blood fatty acids begin to increase about two hours after a meal and reach a peak about three to five hours later, when they then decline. There is evidence that the phospholipids and sterols play a role in the resynthesis of neutral fat, and in its mobilization and deposition in the depots. There is further evidence that free fat can enter the fat cells of the reserve depots unchanged,

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TABLE I

Effect of Deep Massage Upon Fatty Acids and Cholesterol of the Blood in Obesity. One-Half Hour Deep Massage Over Obese Areas.

CASE NO.	FATTY ACIDS			CHOLESTEROL		
	BEFORE	AFTER	PER CENT + OR −	BEFORE	AFTER	PER CENT + OR −
1	362	391	+ 8.0	176	193	+ 9.7
2	384	376	− 2.1	240	230	− 4.2
3	446	421	− 5.6	187	193	+ 3.2
4	397	441	+11.0	181	200	+11.0
5	353	401	+13.6	207	207	0.0
6	411	459	+11.8	183	188	+ 2.7
7	402	390	− 3.0	236	172	−27.2
8	668	691	+ 3.4	274	264	− 3.8
9	361	330	− 8.6	182	176	− 3.3
10	307	365	+18.5	176	166	− 5.7
11	365	313	−14.2	150	162	+ 8.0
12	218	200	− 8.3	176	193	+ 9.7
13	224	227	+ 1.3	240	272	+13.3
14	341	354	+ 3.8	215	222	+ 3.3
15	409	414	+ 1.2	187	200	+ 6.9
16	388	367	− 5.4	193	208	+ 7.8
17	270	284	+ 5.2	162	166	+ 2.5
18	378	361	− 4.6	200	206	+ 3.0
19	362	390	+ 7.8	230	222	− 3.5
20	498	504	+ 1.2	240	230	− 4.2
Average	377	384	+ 1.8	202	204	+ 1.0

TABLE II

Effect of Local Heat Upon Fatty Acids and Cholesterol of the Blood in Obesity. Areas Heated by Means of Induction Coils and Short Wave Therapy.

CASE NO.	FATTY ACIDS			CHOLESTEROL		
	BEFORE	AFTER	PER CENT + OR −	BEFORE	AFTER	PER CENT + OR −
21	485	555	+14.4	300	300	0.0
22	385	339	−11.9	240	200	−16.6
23	457	585	+28.1	260	214	−17.7
24	505	487	− 3.6	231	240	+ 3.9
25	407	418	+ 2.7	230	222	− 3.4
26	390	404	+ 3.6	206	222	+ 7.8
Average	438	465	+ 6.2	245	233	− 4.9

but to gain access to the tissue cells, such as the liver, the fatty acids must first be converted to a phosphatid by the addition of phosphorus and nitrogen.

In a former study, the writer demonstrated an increase in the total blood fats following ether anesthesia.[1] This was attributed to the solvent effect of ether and its ability to carry fat with it into the blood stream. Murlin and Riche[2] reported an increase in blood fats after exercise in 1916. In our study active exercise was not used, but merely vigorous heavy massage for one-half to one hour over areas like the thighs and hips of obese individuals where fat was particularly in evidence. With the patient in the postabsorptive state, blood was taken before and immediately following the massage or thermal treatment. It was then analyzed for total fatty acids and cholesterol. Total acetone bodies were done on the early experiments, but were discontinued because they were uniformly within normal limits. The findings for fatty acids and cholesterol are shown in Tables I-IV.

TABLE III

Effect of Local Heat Followed by Deep Massage Upon Fatty Acids and Cholesterol of the Blood in Obesity. Heat by Induction Coil.

CASE NO.	FATTY ACIDS			CHOLESTEROL		
	BEFORE	AFTER	PER CENT + OR −	BEFORE	AFTER	PER CENT + OR −
27	442	460	+ 4.6	187	167	−10.7
28	387	358	− 7.5	250	230	− 8.0
29	403	390	− 3.2	260	273	+ 5.0
30	401	331	−17.5	240	230	− 4.2
Average	408	385	− 5.9	234	225	− 3.8

TABLE IV

Effect of Artificial Fever Upon Fatty Acids and Cholesterol of the Blood in Obesity. Blood Taken After Approximately Thirty Minutes' Immersion in Tank of Hot Water With Oral Temperature at 101° F.

CASE NO.	FATTY ACIDS			CHOLESTEROL		
	BEFORE	AFTER	PER CENT + OR −	BEFORE	AFTER	PER CENT + OR −
31	335	390	+13.4	213	222	+ 3.9
32	329	400	+21.6	230	240	+ 4.4
33	382	375	− 1.8	250	230	− 8.0
34	397	404	+ 1.8	171	174	+ 1.7
35	384	388	+ 1.0	187	187	0.0
36	347	339	− 2.3	193	181	− 6.2
37	438	422	− 3.7	300	260	−13.3
Average	373	388	+ 4.1	221	219	− 0.9

COMMENT

There is some obscurity about the means by which fats are mobilized from their depots from the production of heat and energy. It seems reasonable to believe that mechanical and thermal means, such as were used in these experiments, might cause a sufficient added increment to the blood stream to be detectable by chemical analysis. This was not proved to be the case. Several explanations of our negative findings suggest themselves: (a) lipoids cannot be mobilized from their depots by the means employed; (b) the amount mobilized was too small to be detectable when added to the relatively large volume of circulating blood; (c) a sufficient time interval was not allowed to permit the lipoids to reach the blood in greatest concentration; (d) fluids were simultaneously mobilized from the tissues which, by blood dilution, obscured the added increment of lipoids. Further studies will be necessary to determine which explanation is correct.

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SKIN REACTIONS*

VI. A Simple Micromethod for the Assay of Histamine in Mammalian Blood

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INTRODUCTION

In the absence of high concentration of electrolytes, histamine in dilutions as high as 1:5,000,000 may be detected by wheal formation when forced into the human skin by means of the direct electrical current.[1] That is, if some absorbent material is wet with a solution of histamine and made the positive pole by means of a suitable metal contact, at comparatively low current densities, sufficient histamine is carried into the skin by ionic and electro-osmotic transport to produce its characteristic effect. In view of the difficulties attendant both upon the chemical determination of histamine and upon biological assay by means of the contraction of smooth muscle, a simple method to detect histamine in the presence of blood would be desirable. This method, based upon the response of the skin by wheal formation subsequent to electrical transport of histamine into the skin, has now been developed and forms the subject of this communication.

METHOD

Source of Current.—The apparatus previously described by Abramson and Alley[1] may be used if it is desired to make more than one determination at a time. The equipment consists merely of a 45 volt B battery which can be tapped through one or more potentiometers. Milliammeters reading from 0 to 5 Ma. are in series in the circuit of the subject.

Electrode Materials.—The absorbent material containing the solution suspected of having histamine is placed in contact with the skin of the anterior aspect of the forearm, a metal electrode being employed to establish contact with the source of current. The success of the method depends upon its reproducibility; and the reproducibility in high dilutions depends largely upon the choice of the material for the electrodes. After some experimentation, the copper gauze electrode described by Abramson and Alley was discarded in favor of an electrode of light copper foil, curved slightly so that it rested evenly on the absorbent electrode. The pressure was thus distributed uniformly because the foil is thin enough to yield to pressure before the skin itself is appreciably distorted. Pressure on the developing wheal may retard or prevent its appearance.

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The following absorbent materials were tested: absorbent cotton, Canton flannel, blotting paper, and asbestos paper. Electrodes (having an area of 1 sq. cm.) of these materials were examined with special reference to their usefulness at low concentrations. Although the blotting paper is advantageous as far as uniformity and ease of handling are concerned, better distribution of pressure and electrical contact was obtained with absorbent cotton. Surprisingly small volumes of liquid are needed to wet 1 sq. cm. of cotton or blotting paper between 1 to 2 mm. thick. The final technique employed required 0.1 c.c. of liquid for each determination, although smaller volumes of liquid could be used if the area of the electrodes was correspondingly reduced. Whatever absorbent material is used, however, must be tested for the presence of contaminating (wheal producing) substances. Observations were usually made at the end of ten or fifteen minutes, although the wheals were frequently observed for much longer periods. The investigator will find it desirable to standardize his own technique. Thus, with high dilutions, it may be necessary to observe the maximum effect, rather than the effect at a given time.

Position of Negative Electrode.—The effect of the position of the negative electrode on the manner in which the wheal spread was studied in the following way. The negative electrode was either held in the hand or placed immediately above, below, or opposite the positive electrode, with no obvious influence on the direction of the spread of the wheal. In the experiments reported here, the negative electrode was usually held in the hand of the same arm used.

It was also of interest to see if the extent of pseudopod formation depended primarily upon the distribution of the lines of electric current. Duco household cement painted on the skin acts as an efficient insulator and prevents wheal formation following histamine iontophoresis over a protected area. The film of cement is sufficiently transparent to observe many phenomena occurring beneath it. Histamine wheals were produced on the anterior surface of the forearm, with frames of Duco cement about the absorbent cotton. In this way the lines of current entering the skin could not pass through the Duco frame. Wheals produced under these conditions developed pseudopods which could be seen to spread beyond the inner boundary of the insulating frame, and extended widely beyond it, with higher concentrations of histamine. In other words, the spread of the wheal was independent of the lines of current. These, as well as similar experiments, lend support to the point of view expressed by Abramson and Engel,[2] following the observation of McMaster and Hudack, that wheal formation of this type is due essentially to a convection of histamine through the lymphatic capillaries, with escape of histamine back into the interstitial tissues to affect the blood capillaries locally at the site of escape.

Limits of the Method.—Since the detection of the presence and quantity of histamine by the method of iontophoresis is a biological assay, the absolute quantity of wheal-producing substance cannot be measured. However, the method does not suffer from the restriction imposed by the destructive processes of chemical procedures necessary in the chemical methods. Extracts of blood having activities like that of histamine have been extensively investigated. Although Harris[3] employed simple alcoholic extracts, Best and McHenry[4] boiled the blood with hydrochloric acid. Code[5] has maintained that boiling with acid

might lead to secondary reactions. He developed methods free from this objection. As far as we have been able to discover, assays have previously been performed by observing the effects of extracts on the contraction of smooth muscle. In the absence of electrolytes, about one part in five million of histamine can usually be easily detected by the appearance of scattered papules on the skin of the forearm. Mammalian blood is approximately equal in electrolyte content to M/7 sodium chloride solution. As pointed out by Abramson and Alley,[1] salts may, and in sufficient quantity do, diminish the histamine transported not only because of a partition of current carried between positively charged histamine and other ions, but also because of the depressing effect of salts on the electrokinetic potential of the skin. For this reason, the lowest concentration of histamine which can be detected lies between 1:2,000,000 and 1:5,000,000.

TABLE I

WHEAL PRODUCTION BY DEFIBRINATED BLOOD CONTAINING VARIOUS QUANTITIES OF HISTAMINE (UNWASHED ABSORBENT COTTON)

HISTAMINE CONCENTRATION	RESULT
0	No wheal
1:10,000,000	No wheal
1:1,000,000	Flat, fairly confluent wheal
1:1,000	Spreading wheal with pseudopods

EXPERIMENTAL

Recovery of Histamine From Human Blood.—Preliminary experiments with human blood obtained from pricking the finger tip showed that capillary blood did not contain substances which produced a wheal with our technique. The detection of histamine in defibrinated blood is given in Table I. The data in the table were obtained by making dilutions of histamine in freshly defibrinated blood. With 1:1,000,000 histamine, in the presence of blood, a well-defined, fairly confluent wheal was obtained under the conditions described in Table I. This typical experiment also disclosed that insufficient whealing substance formed in the blood standing at room temperature after one hour. Further, histamine may readily be detected almost quantitatively if present in human blood in the concentrations described, for the type of wheal obtained corresponds approximately to that usually observed with simple solutions of histamine having similar quantities of electrolytes.

In addition to the experiment described in the foregoing, the following samples of blood or materials derived from blood (one series of experiments) did not contain sufficient whealing substance to produce a wheal with our technique. Cotton electrodes and a current density of 0.5 Ma. for five minutes were used.

(1) Oxalated human plasma.
(2) Buffy coat from human plasma.
(3) Buffy coat frozen and then thawed.
(4) Red cells (washed) following freezing and thawing until hemolyzed.

If these experiments are subsequently confirmed, the histamine liberated and remaining in the solution during the freezing and thawing of human blood cells is probably less than one part in three million.

Recovery of Histamine From Rabbit Blood.—The whole blood of the rabbit apparently contains a substance capable of being transported electrically

to produce a wheal in normal human skin.[3] In 18 experiments with whole rabbit blood, using 8 different rabbits in 5 different individuals apparently not sensitive to rabbit serum, definite wheals were obtained in 16 instances under conditions identical with those producing no effect with saline or human blood. If sensitiveness of these individuals to rabbit blood itself can be excluded, the production of a wheal under these circumstances is confirmatory evidence of the data of Codes that normal rabbit blood contains approximately one part in one million of histamine or of a histamine-like substance. In all of the instances in which wheals were produced by rabbit blood, a 1:10 dilution of the blood with saline resulted in a complete loss of the wheal-producing properties of the solution when administered by the method in question. It was concluded from these experiments that the study of the effects of blood on the wheal-producing properties of histamine might be investigated under the conditions described in the foregoing if precautions were taken to dilute the blood to one part in ten of saline.

Histamine was dissolved in distilled water and 0.1 c.c. of the solution was added to cotton squares. These were dried for one and one-half hours in an oven at 60° C. and then five hours in a calcium chloride desiccator. The effect of whole, nonoxalated, rabbit blood on these cotton squares containing varying concentrations of histamine is given below.

TABLE II

HISTAMINE CONCENTRATION	RESULT
0; 1 part blood, 10 parts saline	No wheal
0	Fairly confluent wheal lasting about thirty minutes
1:5,000,000	Fairly confluent wheal lasting about thirty minutes
1:500,000	Fairly confluent wheal lasting one hour; slight spread
1:150,000	Large, fairly confluent wheal lasting two and one-half hours; marked spread with many pseudopods

TABLE III

WHEAL FORMATION BY RABBIT'S BLOOD (DILUTED 1:10 WITH PHYSIOLOGIC SALINE) CONTAINING VARIOUS QUANTITIES OF HISTAMINE

HISTAMINE CONCENTRATION	RESULT
0	No wheal
1:4,500,000	Small discrete papules
1:1,500,000	Fairly confluent flat papules
1:150,000	Well-marked spreading wheal

Although whole rabbit blood contains a wheal-producing substance, histamine was readily detected: (a) when histamine was added to rabbit's blood; (b) after rabbit's blood containing histamine was diluted with saline; and (c) in rabbit's blood following intravenous administration of histamine.

(a) To illustrate the first point note the data in Table II which were obtained by adding whole rabbit's blood (nonoxalated) to dry squares of cotton (1 cm.^{2}) containing various quantities of histamine. These were prepared by adding 0.1 c.c. of histamine solutions of various concentrations to cotton squares

(1 cm.^{2}) and drying the cotton at 60° C. for one and one-half hours and in a desiccator for five hours. Blood was added previous to each experiment.

(b) If oxalated rabbit blood is added to saline containing histamine and the mixture is diluted with saline to higher dilutions, the histamine can be readily assayed. An experiment of this type is depicted in Table III. The initial dilution of blood was 1:10 and that of the histamine 1:10,000. The first dilution of the blood resulted in removing the effective wheal-producing substance. Subsequent dilution by saline permitted the whealing effects to be observed in dilutions up to about 1:4,000,000.

(c) That histamine may be readily detected in rabbit's blood is perhaps demonstrated more clearly by the following experiment. A rabbit, weighing 2 kg., was given 2 mg. of histamine intravenously by a marginal vein of the ear. While the animal was dying, blood was obtained by cardiac puncture. Before the injection of histamine, assay of a 1:10 dilution of blood in saline produced no wheal as usual. After histamine, the following results were obtained (current density 0.5 Ma. for five minutes):

DILUTION OF BLOOD	CHARACTER OF WHEAL
1:10	Irregular confluent wheal lasting thirty-five minutes
1:100	Slight, faint, discrete papules
1:1000	No wheal

According to this assay, the papules observed with a 1:100 dilution of blood represented a dilution of histamine close to 1:5,000,000. On this basis the dilution in the blood was 1:50,000. Indeed, the undiluted blood of the animal in question after the administration of histamine did give a large, persistent, confluent, spreading wheal by iontophoresis. If the blood volume of the rabbit is taken as 200 c.c., the immediate dilution (death occurred within two minutes) would be 1:50,000, which corresponds to that dilution observed in the assay.

In another rabbit, which was killed by 0.7 mg. of histamine intravenously, the blood obtained by cardiac puncture produced minute, discrete papules in a 1:10 dilution in saline after five minutes, with a current density of 0.5 Ma. The 1:100 dilution was negative. If the blood volume of the rabbit was 200 c.c., the immediate dilution of the histamine in the blood was about 7:2,000,000 or 1:3,000,000, approximately. The size and type of wheal observed was roughly in agreement with this calculation.

TABLE IV

HISTAMINE ADDED TO GUINEA PIG BLOOD AND DILUTED WITH SALINE

HISTAMINE CONCENTRATION	RESULT	
	FIVE MIN.	TEN MIN.
0	No whealing	No whealing
1:4,500,000	Scattered discrete, slightly elevated papules	Scattered discrete, slightly elevated papules
1:900,000	Confluent central wheal surrounded by discrete papules	Tendency toward confluence and somewhat flatter
1:100,000	Confluent wheal larger than area of the electrode; discrete papules on periphery	Confluent flat wheal

Recovery From Guinea Pig Blood.—Oxalated blood from two adult guinea pigs by heart puncture did not produce wheals with our method. Guinea pig blood (oxalated) did not prevent the histamine from exercising its usual whealing effect. Further, histamine was also readily detected if guinea pig blood containing histamine was diluted with isotonic saline (Table IV).

To determine if histamine could be detected in guinea pig blood following intraperitoneal administration, 4.8 mg. of histamine was given to a guinea pig weighing 580 gm. While the animal was dying, blood was withdrawn by cardiac puncture. The oxalated blood (which on two previous occasions showed no whealing), now produced fine discrete papules, corresponding to about 1:5,000,000 histamine. The 1:10 dilution produced no wheal. In a similar experiment which differed from the preceding, the guinea pig was practically symptom free fifteen minutes after the injection and subsequently recovered. There was no whealing with the oxalated blood. According to our assay, the dilution of histamine in the blood was less than 1:5,000,000 in this instance.

DISCUSSION

Since the blood of the rabbit killed by less than 1 mg. of histamine given intravenously contained considerable quantities of histamine, it follows that only a small part of the histamine originally administered was needed to produce death. That is, an equilibrium of the following type may be set up:

$$\text{Histamine (blood)} \rightleftarrows \text{Histamine (tissues)} \rightleftarrows \text{Histamine (destroyed)}$$

This equilibrium would exist in favor of the first term according to the experiment just cited. Unpublished data obtained with Lubkin during a study of the histamine wheal indicated that following wheal formation by iontophoresis of histamine, it can be recovered by reversed iontophoresis for as long as forty minutes under certain conditions.[6] The type of equilibrium in the dermis analogous to the preceding has been studied and will be described in a subsequent communication.

With the technique described in the foregoing, the skin of most subjects responded by discrete papules to a concentration of histamine close to 1:5,000,000. The marked differences in sensitiveness to histamine supposed to exist for the skin surfaces of different individuals were not noted. Our impression is that there was more variation in response to the electric current alone (controls) than to the histamine administered. The erythema produced by the current alone was usually fairly well localized to the electrode area, whereas that produced by histamine was always extended radially from it.

Horsfall[7] has observed a phenomenon closely related to the observation that wheals can be produced by the iontophoresis of rabbit blood. Horsfall found that intradermal injections of rabbit antipneumococcus serum was followed by whealing in individuals not immunologically sensitive to rabbit serum. Even though the skin test may be positive to these sera, they may be administered intravenously without anaphylactic responses. Since we have shown that the wheal-producing agent is readily introduced into the skin by electrophoresis from the positive pole, it could be inferred that the whealing substance is positively charged. However, this is by no means a necessary conclusion. As noted

hitherto, histamine has been transported electrically into the skin even when it was dissolved in aqueous solutions of alkali the pH of which was sufficient to almost completely suppress the ionization of the histamine salt. Under these conditions the undissociated histamine is transported by electro-osmosis. It is of some importance to determine in more detail the nature of the whealing substance in rabbit sera.

There are many body fluids and tissues which are supposed to contain histamine in considerable quantities. The method described here can probably be adapted for the determination of histamine in gastric contents, for extracts of the skin and other tissues. The method would provide, for example, a simple procedure for following the rate of disappearance and fate of histamine in animals like the rat, which are insensitive to massive doses.

SUMMARY

1. Previous investigations have shown that wheal formation by histamine following electrical transport into the human skin permits the detection of histamine in dilutions as high as 1:5,000,000.

2. A standard, more reproducible method of applying the positive electrode has been developed.

3. Wheal formation was essentially independent of the position of the negative electrode and of the lines of current at the positive pole.

4. After addition of histamine it was detected in human, rabbit, and guinea pig blood in dilutions between 1:2,000,000 and 1:5,000,000 by the electrophoretic method of producing wheals.

5. Using this method, it was found that rabbit blood itself produced wheals in five individuals. This capacity to produce wheals disappeared when the blood was diluted ten times. Human and guinea pig blood did not contain a wheal-producing substance. Histamine was easily detected in the cardiac blood of rabbits killed by intravenous administration of histamine.

6. The bearing of the data obtained thus far on the nature of the equilibrium between histamine and the tissues is briefly discussed.

We are indebted to Mrs. Margery Engel for assistance in these experiments.

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*Dr. Peyton Rous has informed one of us (H. A. A.) that Ebbecke (Arch. f. d. ges. Physiol. **195**: 300, 1922) was the first to employ histamine iontophoresis.

†One of us (H. A. A.), working with Gorin and Engel, found that histamine may be recovered from the skin for at least two days subsequent to histamine iontophoresis. Its presence in the skin may frequently be shown for four days. This was not so following ordinary intradermal administration.

LABORATORY METHODS

A METHYLENE BLUE STAIN FOR SECTIONS OF FORMALIN-FIXED MARROW*

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THE following methylene blue stain is recommended for sections of formalin-fixed bone marrow. The method is simple, rapid, and gives constant results. It depends upon the simple principles of deformolization, sensitization, overstaining, and selective differentiation.

METHOD

Suitable pieces of bone marrow are fixed in 10 per cent formalin solution (4 per cent formaldehyde), decalcified in dilute mineral acid, washed in water, hardened and dehydrated in alcohol, cleared in xylol, and embedded in paraffin. In this laboratory 20 per cent nitric acid is used as the decalcifying agent, and the calcium salts are removed under vacuum. This reduces the time needed for decalcification, keeps the tissue in motion, and inhibits the formation of large bubbles of carbon dioxide gas which damage the tissues. Alternatively the bone may be decalcified in the sodium citrate, formic acid mixture suggested by Evans and Krajian.[1] Sections should be cut 4 to 6 microns thick, affixed to cover slips or slides, and dried in the oven. If the glassware is thoroughly clean and free of grease, the sections will adhere to the glass without egg albumen. The paraffin is removed with xylol, and the sections are brought into water in the usual manner.

Deformolization.—The formalin fixative is removed by immersing the sections for one minute in 10 per cent ammonia solution (one part of 28 per cent ammonia and nine parts of water) kept in a covered dish. Longer immersion is undesirable because the sections may become detached. The ammonia gradually loses its strength and should be renewed as required. (Zenker-fixed material may be used. It does not require the ammonia bath; instead, it should be treated with Lugol's iodine, followed by sodium thiosulfate solution and a thorough wash in water to remove the precipitates of mercury salts.)

Sensitization.—The sections are washed in water to remove the ammonia and mordanted for three minutes in a mixture consisting of equal parts of Zenker's fluid (without acetic acid) and saturated aqueous solution of copper sulfate.

Staining.—After a thorough wash in two changes of water, the sections are stained for one and a half to three minutes in the following mixture:

1 per cent aqueous solution of eosin Y	one part
1 per cent aqueous solution of phloxin	three parts

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They are then washed in water and stained for four minutes in Loeffler's alkaline methylene blue, heated to 37° C.

Differentiation.—The sections are washed in water and differentiated in absolute alcohol. Differentiation should be controlled by microscopic examination. As soon as differentiation is complete, the sections are cleared in xylol and mounted in balsam.

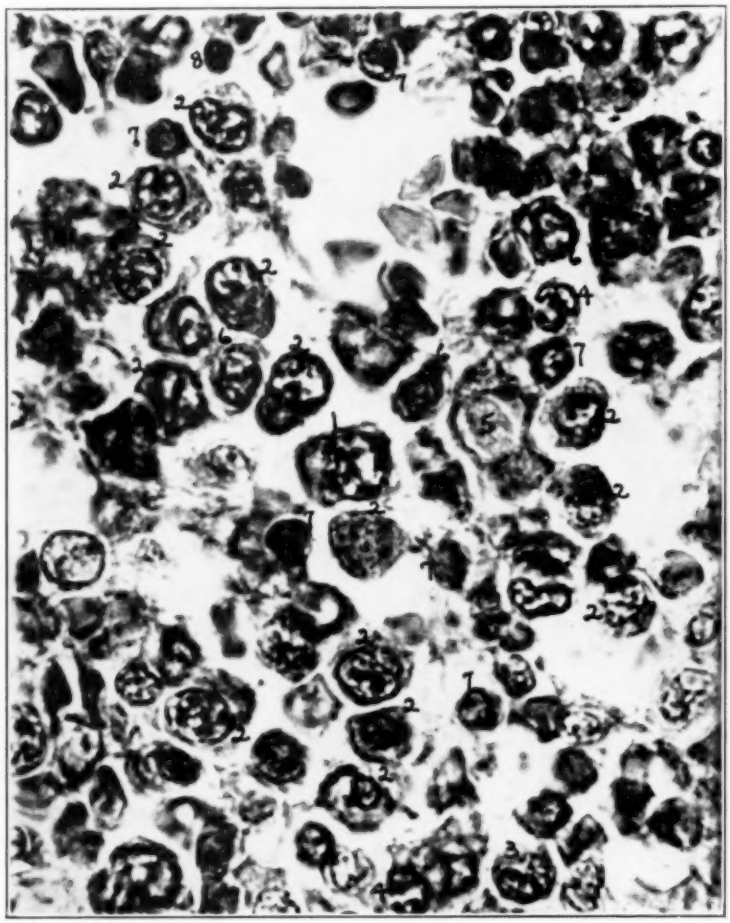

Fig. 1.—Section of sternal marrow from a case of myelogenous leucemia. Tissue was fixed in formalin, decalciffied with dilute nitric acid, and stained with methylene blue, eosinphloxin. Magnification 740 diameters. *1*, premyelocyte; *2*, neutrophilic myelocytes; *2a*, dividing myelocytes; *2b*, eosinophilic myelocytes; *3*, metamyelocytes; *4*, young polymorphonuclear leucocytes; *5*, endothelial cells; *6*, macronormoblasts; *7*, intermediate normoblasts; and *8*, micronormoblasts. A majority of the cells are myelocytes, but there are also a number of normoblasts.

Results: The marrow cells, stroma, and capillaries are all stained very precisely (Fig. 1). The granules of the myeloid cells stand out sharply. The normoblasts have a faint red cytoplasmic border; the immature cells a pale blue cytoplasm. The nuclei of all cells are sharply outlined in varying shades of blue, so that identification of the various cells is greatly simplified. This is an important consideration now that diagnosis of biopsy specimens of marrow is requested so frequently.

Comment: The stain does not fade if kept away from strong daylight. Exposure to direct sunlight causes the methylene blue stain to fade completely at the end of two weeks, but the light of a 15 Watt substage microscope lamp has no visible effect at the end of six weeks' continuous exposure. While primarily devised for sections of bone marrow, the method is equally suitable for soft tissues.

The time periods advised give optimum results, but may be varied. Differentiation in alcohol is the only step requiring careful attention. With a

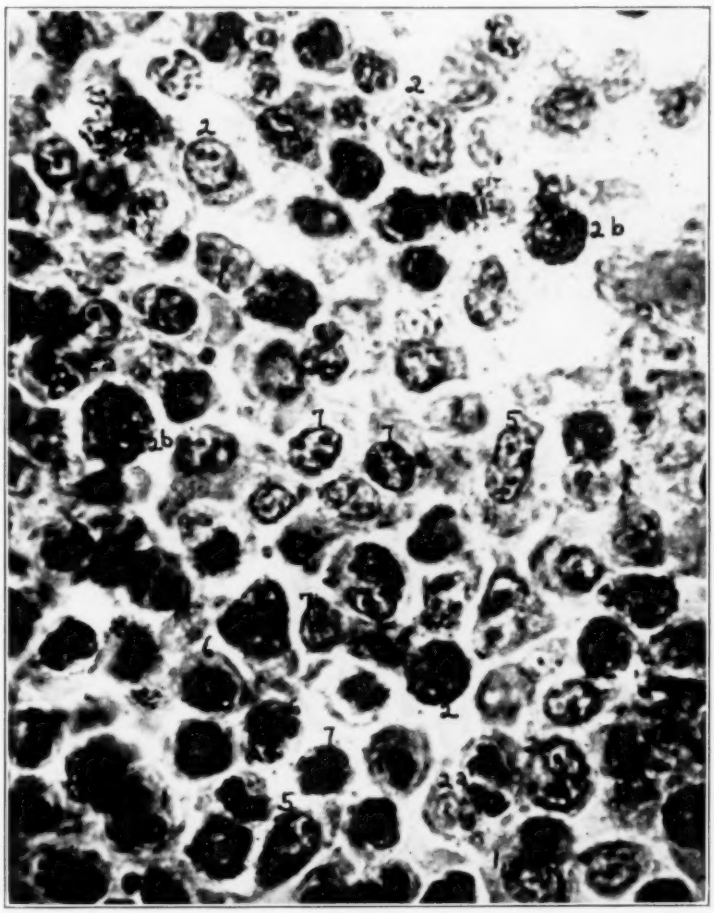

Fig. 2.—Another field from the same section, showing two dividing cells and the coarse granules of eosinophilic myelocytes.

little experience, however, the correct stage at which differentiation should be arrested may be recognized by the unaided eye. The methylene blue is removed slowly by absolute alcohol (much more rapidly by 95 per cent alcohol), while the cytoplasmic stain is alcohol fast. Neither eosin nor phloxin—alone or in succession—gives as bright or precise a stain as the eosin-phloxin mixture. This mixture keeps indefinitely and may be used repeatedly. Similarly, copper sulfate by itself is only a fair mordant and its effectiveness is greatly increased when it is combined with Zenker's fluid.

This mixture also may be used repeatedly. Occasionally the sections become loose in the ammonia bath. This may be prevented by floating the sections on to a warm 0.25 per cent aqueous solution of gelatin, as they are cut. They are then transferred to slides or cover slips and dried in the oven in which is kept a small unstoppered bottle of full strength formaldehyde.

SUMMARY

A methylene blue stain, suitable for sections of formalin-fixed bone marrow (or soft tissue), is described.

The method may be summarized as follows:

1. Fix tissues in 10 per cent formalin, decalcify in dilute mineral acid, wash in water, harden and dehydrate in alcohol, clear in xylol, and embed in paraffin.

2. Cut sections 4 microns thick, transfer to cover slips or slides, dry in the oven, deparaffinize in xylol, dehydrate in graded alcohols, and place in water.

3. Ten per cent ammonia (2.8 per cent ammonia), one minute. (Zenker-fixed material does not require the ammonia bath, but should be treated with Lugol's iodine for five minutes, washed in water, decolorized in 5 per cent sodium thiosulfate, thoroughly washed in water, and then mordanted and stained as described below.)

4. Wash in tap water.

5. Mordant in a solution composed of equal parts of Zenker's fluid (without acetic acid) and saturated aqueous solution of copper sulfate, three minutes.

6. Wash in two changes of tap water.

7. Stain for two minutes in an eosin-phloxin mixture:

1 per cent aqueous solution eosin Y	one part
1 per cent aqueous solution phloxin	three parts

8. Wash in tap water.

9. Stain in Loeffler's alkaline methylene blue, heated to 37° C. four minutes.

10. Wash in tap water.

11. Differentiate carefully in absolute alcohol, clear immediately in two changes of xylol, and mount in balsam.

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PHOTOELECTRIC RECORDING OF THE PULSE AND OF OTHER OSCILLATORY MOVEMENTS WITH THE ELECTROCARDIOGRAPH*

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Marrazzi[1] called attention in 1935 to the fact that the photoelectric cell could be conveniently used for the detection and recording of movement. He suggested the possibility of wide application. It seemed worth while to extend his suggestion to the recording of the pulse. The technique described below differs in certain essential details from that of Marrazzi.

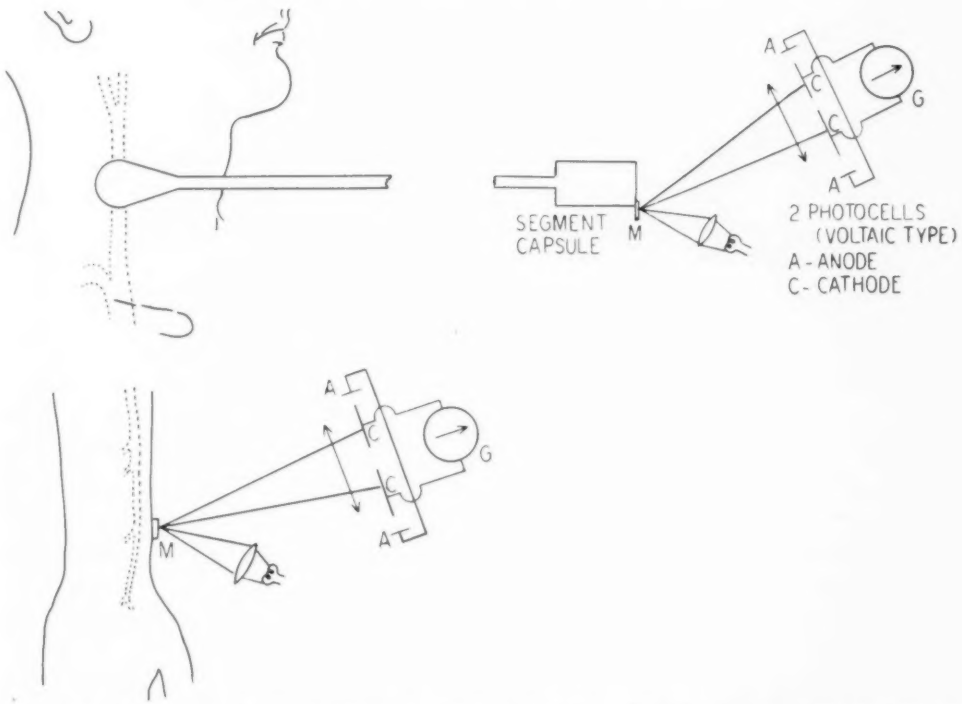

Fig. 1.—Schema of arrangements for photoelectric recording of the pulse with the string galvanometer without amplification. The upper figure shows the recorder using the Frank segment capsule; the lower figure, using a mirror cemented to the skin over the artery.

Two photoelectric cells of the voltaic type (the General Electric Light Sensitive Cells are satisfactory for this purpose) are mounted side by side (Fig. 1). Their cathodes are connected to the lead of the electrocardiograph, while their anodes are connected together. If equal amounts of light fall simultaneously on the two cells, the currents generated in each oppose and nullify

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each other. This eliminates the need for devices supplying opposing potentials to prevent excessive current outputs by the photocell from reaching the string. If a beam of light is now caused to fall on both cells, its oscillations from one cell toward the other (as indicated by the double arrow in Fig. 1) will be reflected in corresponding current oscillations which are in turn recorded by the electrocardiograph. This fact is made use of in recording the pulse in the manner indicated in Fig. 1, employing either a Frank segment capsule or a mirror cemented to the skin. The light from a pencil type of flashlight or an ophthalmoscope is focused on the mirror and reflected back to the two photocells, so that it falls partly on each. The oscillations of the mirror, with the pulse causing corresponding oscillations of the reflected light beam, are thus recorded by the electrocardiograph. It is unnecessary to control carefully the intensity of the illumination or the distance of the photocells from the mirror, since these things

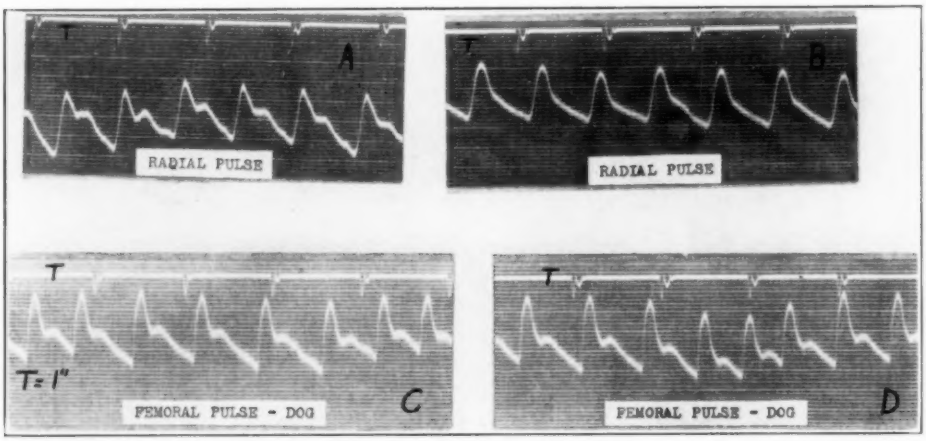

Fig. 2.—The arterial volume pulse recorded photoelectrically with the aid of a mirror cemented to the skin over the artery (*A*, *B*, *C*). *A*, young male adult; *B*, male, aged 75 years; *D*, the femoral volume pulse recorded with a photoelectric skin plethysmograph.

affect the two photocells almost equally. The relative amounts of light falling on each photocell are also unimportant. This renders unnecessary particular care in alignment, since the mirror's oscillations are the only important influence causing variations in the photoelectric current delivered to the electrocardiograph.

Illustrative records of the arterial volume pulse so recorded are supplied in Fig. 2. The lower right record taken with a photoelectric skin plethysmograph (the details of which will be reported elsewhere) is inserted for purposes of comparison with the lower left record taken with a mirror cemented to the skin. The essential identity of the two records is apparent.

Variations in the sensitivity of the recording are conveniently provided by the shunts in the string galvanometer or by variations in the intensity of the illumination, thus eliminating the necessity for long opical lever arms. The application to recording of other oscillatory movements, such as those exhibited by optical manometers of the Hamilton or Wiggers type, is obvious.

SUMMARY

Photoelectric techniques are described which provide in a simple inexpensive manner for direct recording without amplification of the arterial or venous pulses, or of other oscillatory movements by the unmodified electrocardiograph.

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THE RESPONSE TO LIVER EXTRACT OF EXPERIMENTALLY INDUCED (TYPHOID) ANEMIA IN RABBITS: NEGATIVE RESULT*

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EXPERIMENTS recently reported by Wolf, Weber, and Kröger[1] suggest that anemia induced in rabbits by injections of deproteinized typhoid bouillon concentrate may respond in a specific manner to liver extract, and thus may constitute a method of assaying and standardizing the hematopoietic liver principle. Their "typhoid-toxin" is obtained by allowing a typhoid bouillon culture to incubate until "sterile by autolysis," followed by precipitation of the proteins with colloidal iron and concentration of the filtrate in vacuo. This material is then injected subcutaneously into rabbits, the effective daily dose being from 1.5 to 2.0 c.c. per kg. of body weight. Because the toxin is an apparently painful irritant, preliminary local anesthesia (novocain) is recommended. Prompt anemia with depression of leucocytes, platelets, and reticulocytes is said to follow daily injections of the toxin. Recovery on discontinuing injections is slow and imperfect; the concomitant reticulocyte response is irregular, low in magnitude, and variable in type. Death frequently ensues before severe anemia develops.

According to these authors if the rabbits are treated beforehand or concomitantly with daily parenteral liver extract, a different result is obtained. Anemia still appears, but the animals invariably survive and, despite continued toxin (and liver) administration, a reticulocyte crisis (34 per cent in one experiment) is said to occur with subsequent recovery of erythrocyte, hemoglobin, leucocyte, and platelet values. The reticulocyte crisis is said to reach a peak between the eighth and twelfth days. Its similarity in time and character to the classical response to specific treatment of human pernicious anemia is stressed. The authors exhibit three graphs as typifying many similar experiments using various brands of commercial liver extract.

A dependable laboratory method of assaying hematopoietic liver substance has long been a clinical and commercial desideratum. Many attempts have been made, but all have proved either impractical or unreliable. A truly

*From the Hematological Section of the Medical Clinic, Hospital of the University of Pennsylvania. This work was aided by grants to the Hematology Fund from Mrs. Bruce Ford and Mr. Richard P. Brown.

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specific method of bioassay would on a priori grounds seem to predicate the production in the experimental animal of a pathologic state presenting the major hematologic characteristics of pernicious anemia in man. The evidence, presented by the experiments of Wolf, Weber, and Kröger, seems to be far distant from this ideal. Nevertheless, their report of the specific production of a classical reticulocyte crisis by administering liver extract to rabbits rendered anemic by typhoid toxin seemed worthy of repetition.[2]

METHOD

It is unfortunate that the article by Wolf, Weber, and Kröger fails to detail exact procedures in the preparation of the toxin. Their directions were followed, however, as closely as possible. A virulant typhoid strain was incubated at 37° C. for three weeks. The medium was composed of meat infusion

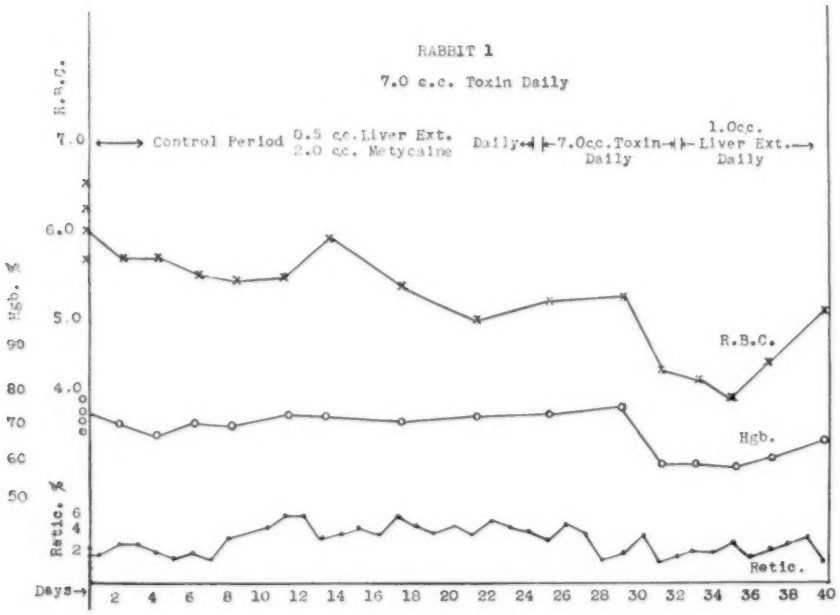

Fig. 1.

broth, Witte's peptone, and 0.2 per cent glucose, pH 8.2. Iron dialysate 5 per cent (Merck) was added in the ratio of 1:2, the resulting percipitate allowed to settle, and the supernatant liquid passed first through paper and finally through Berkefeld N filters. This filtrate was virtually protein free (sulfosalicylic acid and potassium ferrocyanide—acetic acid tests). Five liters of the filtrate were then concentrated at low temperature (25°-28° C.) vacuum distillation. The final product was clear, amber colored, and sterile.*

Three white rabbits, weighing about 2 kg. each, were used. These animals were kept on a basic ration of oats with fresh vegetables added daily. All injections were given subcutaneously, local anesthetic followed by toxin at one site, liver preparation at another site.

*We are indebted to Dr. John S. Lockwood and Dr. Harry M. Va[illegible], of the Department of Research Surgery, for aid in the preparation of this material.

Concentrated liver extract* and reticulogen* were used as sources of hematopoietic liver principle for these experiments. The local anesthetic was metycaine* (1 per cent).

Erythrocyte, hemoglobin (Sahli), and hematocrit (Van Allen) values were determined every second day, leucocyte and platelet counts and blood film examination every fourth day. All counts were performed in duplicate, using certified pipettes and counting chamber. Reticulocyte counts (wet technique) were made daily. Several basal blood examinations were obtained for each rabbit before injections were started.

Rabbit 1 served as a control. Twenty-three consecutive injections of 2.0 c.c. metycaine and 0.5 c.c. liver extract were given. The observed changes in erythrocyte and hemoglobin values were not significant (Fig. 1). Reticulocyte percentage increased irregularly to a maximum of 6.4 per cent on the

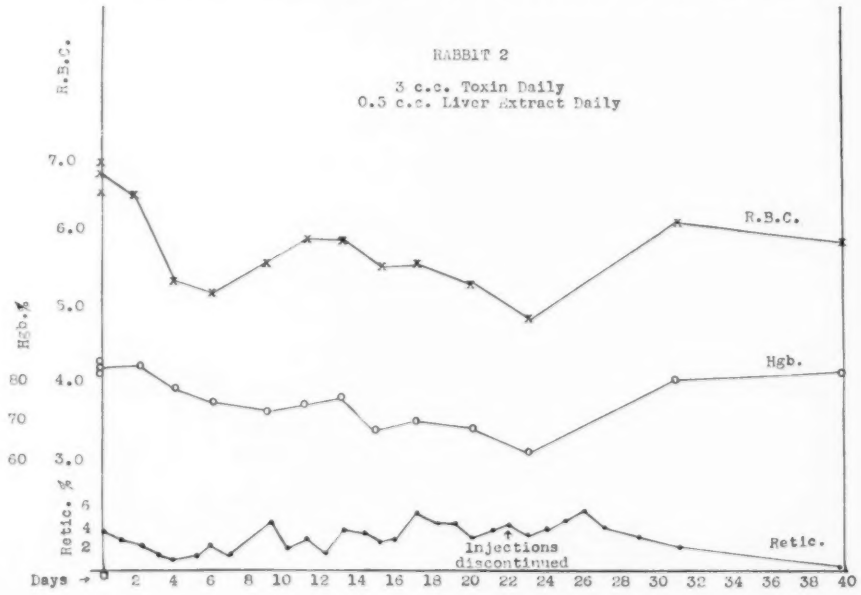

Fig. 2.

seventeenth day. A slight leucocytosis and thrombocytosis occurred. The animal remained entirely well, gained weight, and showed no dermal injury.

Using the same animal and beginning two days after completion of the liver injections (which totaled 11.5 c.c. of concentrated liver extract over a period of twenty-three days), a course of toxin injections was instituted. The animal was given 2.0 c.c. metycaine and 7.0 c.c. toxin (3.1 c.c. per kg. of body weight) daily for nine doses (Fig. 1). There was a prompt, sharp fall in the red cell count and hemoglobin. Reticulocytes did not exceed 6 per cent. Leucocytes and platelets increased. The injections provoked local skin necrosis, induration, and occasional abscesses. The animal appeared listless and lost weight. Immediately after the toxin course, liver extract (1.0 c.c. daily) was again injected. Prompt improvement in red cell count and to a lesser degree in hemoglobin content was seen. There was no significant change in reticulocyte percentage.

*Supplied by Eli Lilly & Co.

Rabbit 2 was injected daily with 3.0 c.c. toxin (1.5 c.c. per kg. of body weight), 2.0 c.c. metycaine, and 0.5 c.c. concentrated liver extract (Fig. 2). A moderate, irregular anemia developed. Reticulocytes rose to a maximum of 6.1 per cent on the seventeenth day. Both erythrocyte and hemoglobin values improved soon after injections were discontinued on the twenty-second day.

Rabbit 3 received twenty-two injections of 5.0 c.c. toxin (2.2 c.c. per kg. body weight), 2.0 c.c. metycaine, and 0.25 c.c. reticulogen (Fig. 3). There was a progressive decline in red blood cell and hemoglobin values. The reticulocyte percentage began to fluctuate sharply on the sixth day; a peak value of 11.1 per cent was reached on the thirteenth day. Only after cessation of injections did the erythrocyte and hemoglobin values improve. The white cell and platelet counts increased soon after injections were begun. Both

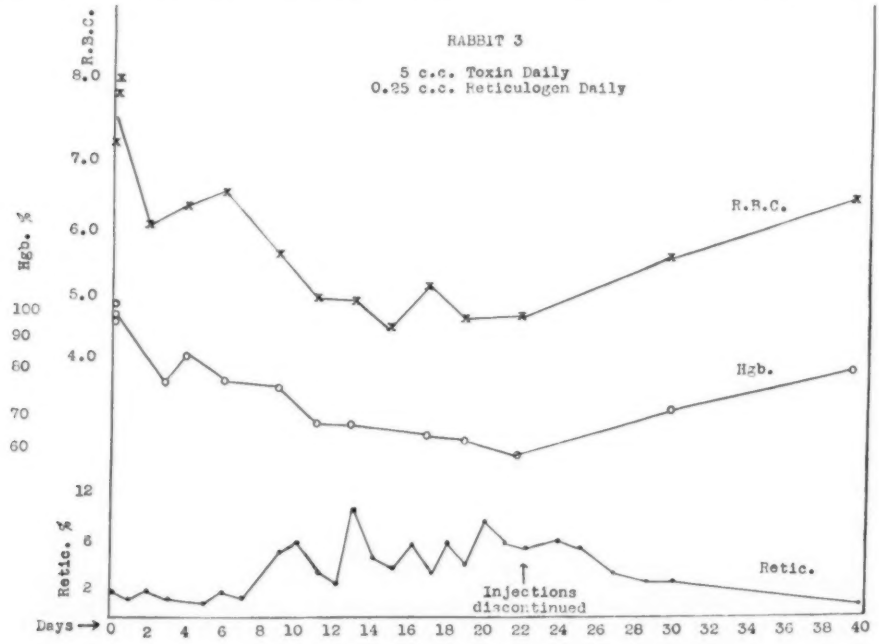

Fig. 3.

animals lost weight, seemed ill, and regularly developed skin necrosis with induration and occasional abscess formation wherever the toxin injection was made.

In none of the toxin-treated rabbits did any significant change in the leucocyte formula appear. There was a moderate increase in the relative and absolute numbers of immature neutrophiles. Anisocytosis, poikilocytosis, and polychromatophilia were slightly more in evidence during anemic phases than during the control periods. Occasional normoblasts were noted. Hematocrit readings paralleled the erythrocyte-hemoglobin fluctuations.

CONCLUSIONS

It cannot be stated with certainty that the anemia produced in the toxin-treated rabbits was due entirely to the marrow-depressing action of typhoid toxin, as suggested by Wolf, Weber, and Kröger. The degree of local tissue

damage produced by the toxin seemed severe enough to be at least in part responsible for the illness and anemia. We were not able to reproduce the previously reported leucopenia and thrombopenia. On the contrary, our toxin-treated animals, as well as the liver extract and metycaine control animal, developed leucocytosis and thrombocytosis.

With the appearance of anemia in the toxin-treated animals there occurred a low grade reticulocytosis, irregular in onset and duration. The rabbit given 5.0 c.c. of toxin and 0.25 c.c. of reticulogen daily exhibited a moderate reticulocyte response, but it was entirely unspecific in type. A well-defined "peak" was not manifested. The control animal, which received at first only liver (and metycaine), showed practically comparable reticulocyte fluctuations.

Our observations do not confirm those reported by Wolf, Weber, and Kröger. Typhoid toxin does not produce an anemia in rabbits which responds uniformly with a reticulocyte crisis to potent hematopoietic liver preparations. The method is unsuitable for assay of such preparations.

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AN INEXPENSIVE SMALL CONSTANT TEMPERATURE BATH

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IN SMALL laboratories where extreme precision in temperature is not required, a constant temperature bath may be constructed at a very small cost.

1. The container is made by securing a two-gallon oil tin, measuring 8 by 6 by 10 inches, free at any garage. For about $1.00 the plumber can convert this to an open-top double-walled vessel, with all walls and bottom double. The air space between the walls varies from ¾ to 1 inch. The conversion is made by soldering bright block tin around the original container.

2. The thermostatic control and heating element can be purchased at any fish and pet supply store. There is quite a choice of fish aquarium temperature regulators. An excellent one, costing about $1.25, consists of thermostat, heating element, and temperature control all in one instrument. This is hung into the water from the edge of the container and is held securely in place by two adjustable metal bands. This is an exceptionally accurate instrument for one costing so little. In a continuous five-day test it kept two gallons of water at a constant temperature of 38° C., with but one-half degree total variation either way.

3. To stir the water in the bath, one may use either one of the two following:

A. At the large chain stores one can obtain for about $1.19 a small electric egg beater made by the Chicago Electric Mfg. Co., and called "handywhip." It consists of a mixing bowl, a sturdy induction motor, and a detachable beater

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or agitator. It should be used in series with a 30 or 40 watt lamp. This prevents overheating and also slows the agitator down to the proper speed. It is a very simple matter to rig up a small series-parallel board of three outlets on a little piece of wood.

B. A fish bowl aerator pump may be used. These sturdy little induction motor pumps cost about $15.00 but can be used for a large variety of aerating and stirring procedures. They have been known to run continuously for three years.

The apparatus is set up in the following manner. A sizable ringstand and base are used. The container is set on the base with its long axis at right angles to the long axis of the base. Water of about the desired temperature is added to near the top, and the thermostat-heater is inserted over the side and adjusted by a little experimentation. A clamp attached to the ringstand supports a thermometer in the water. Another clamp supports the stirring motor and agitator; or, if the pump is used, a glass tube extends down to the bottom. Other clamps support the flasks containing the solutions being tested and incubated.

The induction motor of the egg beater will no doubt please a large number of laboratory workers who will find an infinite variety of uses for it. One can detach the agitator and put on a small propeller and use it in the top of an incubator to secure forced draft ventilation. Here it should be used in series with a 40 watt lamp. It is also a most excellent motor to stir small quantities of inflammable material and solutions as ether and acetone. Since it is an induction motor, there is no danger of sparks; there are no brush replacements, and there is no radio interference. In this laboratory it was used as an inexpensive motor to run a homemade kymograph. On a test run, the motor ran continuously (in series) for three days, without the slightest evidence of overheating.

The total cost of the set-up is as follows:

Container	$1.00
Motor	$1.19
Thermostat heater	$1.25
Incidentals	$0.56
Total	$4.00

(Prices are subject to some variation in different localities.)

THE REFINING OF ANTISERA*

II. Improvements in the Preparation of Refined and Concentrated Pneumococcus and Meningococcus Antibacterial Sera

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INTRODUCTION

A METHOD of refining and concentrating antisera was described four years ago.[1] Since then a number of changes have been introduced as continued experience with the procedures warranted their incorporation. These changes will be described here. The alphabetical sequence given in the former paper in the description of the successive procedures will be used, the paragraphs designated with a definite letter referring to the paragraph with the same letter in the former paper. The changes apply equally to the refining of pneumococcus and of meningococcus antisera.

EXPERIMENTAL PROCEDURE

a. *Preparation of Serum.* Immediately after separation from the clot, 0.3 per cent phenol, in the form of a solution containing three parts of phenol and five parts of ether, and 0.005 per cent merthiolate are added.

b. *Dialysis of Serum.* Dialysis is carried out in a cold room at 7° to 10° C., the running tap water in the tank being at the same temperature. Dialysis is allowed to proceed for six days against a comparatively rapid flow of water. No additional phenol is added during dialysis.

c. *"Acid Protein" Precipitation.* Immediately after dialysis solid sodium chloride is added to the cold serum to N/20, and the pH adjusted to 5.1 with normal acetic acid (γ-dinitrophenol as indicator). This is done in the late afternoon. The material is allowed to stand at 7° to 10° C. overnight. The removal of "acid protein" by filtration through paper pulp and "super cel" is carried out as before.

d. *Preliminary Tests for Antibacterial Substance Preparation.* Four 50 ml. portions are adjusted with N/sodium hydroxide to the pH values 5.9, 6.1, 6.8, and 6.8. The first three are diluted with four volumes of distilled water while the last (pH 6.8) is diluted with six volumes. The precipitates are allowed to settle in the cold, and are then centrifuged and dissolved in 10 ml. portions of 1 per cent sodium chloride solution. Supernatants and dissolved precipitates are tested by the precipitin reaction. The precipitate portions show potency and recovery of material; the supernatants, the optimum hydrogen ion concentration for bulk precipitation not incurring loss.

*From the William Hallock Park Laboratory, Bureau of Laboratories, Department of Health, New York.
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e. *Precipitation of Antibacterial Substance.* No preservative is added to the distilled water used in the precipitation. The precipitated antibacterial substance is allowed to settle overnight in the cold, and finally centrifuged.

f. *Solution of Antibacterial Substance.* The precipitate is dissolved in the smallest practicable volume of a solution of 2 per cent sodium chloride and 0.5 per cent phenol (about one-twentieth of the original volume as a rule). The final antibacterial solution is to contain 1 per cent sodium chloride and 0.35 per cent phenol. The above solution is, therefore, diluted with distilled water containing the calculated additional amounts of sodium chloride and phenol to give the required final volume as determined from the preliminary tests and the solid content desired (usually one-seventh to one-fifteenth of the original volume). The solution is adjusted to pH 6.8 with N/sodium hydroxide if the precipitating pH value was less than this, and 0.005 per cent merthiolate is added. The material is kept in the cold. After twenty-four hours, the preparation is centrifuged to eliminate the coarser particles of separated fibrin material. This procedure facilitates the subsequent filtration through paper pulp and "super cel" (Buchner funnel) and the final passing through a small (6 inches) Berkefeld V filter.

e1. *Alternative Method of Precipitation of Antibacterial Substance.* If an excessive amount of antibacterial substance is found in the supernatants of all the test portions, the material is not precipitated with ammonium sulfate to 50 per cent saturation as described in the previous paper. Instead, precipitation tests are run on portions of serum at the same hydrogen ion concentrations as before, but with the addition of six and eight volumes of water in place of four. This procedure almost invariably causes complete precipitation of antibacterial material with antibacterial-free supernatants. The bulk of the serum is adjusted to the determined hydrogen ion concentration, and the volume of distilled water needed for complete precipitation is added.

NOTES ON THE EXPERIMENTAL PROCEDURE

A. The method of purification and concentration described is applicable mainly to antisera. If the procedure is used with antiplasmas, several difficulties arise. Upon dialysis the intake of water is very large, frequently equal to the original volume of the plasma. This dilution makes the preparation more difficult to handle. Furthermore, the fibrin present adds to the difficulties in the filtration through fine-pored pulp and super cel. Even if these complications are surmounted satisfactorily, so much fibrin is found in the concentrated material that final filtration and Berkefelding is accompanied by appreciable loss.

B. As stated in the former paper, it is believed that dialysis of serum not only eliminates most of the salts but also causes changes in the structure of the proteins. Furthermore, when phenol is added during dialysis, other changes may occur, such as the binding of hematin material to the protein aggregate or particle. This combination is not readily broken down in the subsequent purification, and the final product may have more color than if additional phenol had not been added during dialysis. It has been determined definitely that, even if the phenol which is removed in the dialysis is not replaced, sterility of the product is maintained. The original serum must, of course, be sterile.

C. Formerly, all types other than I and II were allowed to remain at pH 5.1 for only five hours. Now, all sera remain at this pH overnight to allow complete precipitation of "acid protein." This technique results in no loss by inactivation.

D. The preliminary tests to determine the best hydrogen ion concentration for antibacterial substance precipitation are still very essential. However, the pH 6.3 sample is not prepared any more. A pH 6.8 sample diluted with six volumes of water has been substituted for it. If the supernatants of all these tests show no antibacterial protein, the pH 6.8 value is preferred. Appreciably less fibrin is carried down with the precipitating protein at this pH. The final preparation also filters more readily and has a better appearance. A serum, which shows loss when precipitated at any volume (within workable limits) at pH 6.8, is encountered at times. For this reason, the pH 5.9 and 6.1 samples are still prepared.

E. The addition of 0.2 per cent phenol to the precipitating water has been given up as unnecessary. Also, recent work has shown that part of the phenol is adsorbed by the precipitated protein.[2] The material is kept at refrigerator temperature throughout.

F. Preparations which are precipitated at pH 5.7, 5.9, or 6.1, gave clear solutions when dissolved. However, when the pH's of these solutions are changed to 6.8-7.0, a suspension or cloud appears, probably caused by fibrin which had been adsorbed and which is set free by the change in pH. This suspension or cloud becomes more pronounced in the cold.

G. The addition of 0.35 per cent phenol and 0.005 per cent merthiolate, in place of 0.5 per cent phenol, is used to ensure sterility.[3]

DISCUSSION

The changes in the method of concentrating and refining the antibacterial material of sera which are described have given more satisfactory products and better recoveries or yields with a considerable number of preparations. The potencies and clinical efficacies of these preparations do not come within the scope of this paper, except for the fact that the method would necessarily be abandoned if certain standards were not attained.

The use of phenol during the various procedures has been reduced as far as possible. Apparently, changes in the proteins are either induced or increased by phenol, changes which may interfere with the purification or produce other untoward effects. Furthermore, as shown elsewhere,[2] phenol is adsorbed by precipitated proteins in considerable quantities. This adsorbed phenol must be reckoned with in the final products when phenol is added to a definite concentration to ensure sterility. It cannot be too strongly emphasized that phenol estimations should be carried out sufficiently frequently, not only on the final products but also at various stages of the manipulations involved in the refining and concentrating procedures, to control the actual quantities present at all times.

Very little of a definite nature can be added at the present time to the theory of the molecular changes involved in the treatments described in these procedures. That aggregation of molecular units is involved at certain stages

and break down at other stages appear unquestioned, although it is obviously difficult to obtain quantitative evidence of their extent.[4-6]

Reference to the former paper may suffice instead of repeating the discussion already presented of various factors of interest.

SUMMARY

Improvements in the procedure used in this laboratory for the purification and concentration of antibacterial sera, particularly those of pneumonia and meningitis, are presented.

Thanks are due the members of the Pneumonia Division of this laboratory for their constant cooperation, and especially Miss Katherine Blount for carrying out the large number of precipitin tests.

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THE USE OF NONCOOKED, NONSTERILIZED COCONUT MILK AS AN ADDITIONAL NUTRIENT SUBSTANCE IN CULTURE MEDIA*

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IT HAS long been known and appreciated that overheating of culture media impairs their growth qualities. It is even true that in some cases valuable characteristics of the organism are not exhibited in these overheated media.

It is logical to believe, therefore, that the growth quality would be enhanced and the characteristics of organism more accurately demonstrated if an uncooked nutrient were added to cooked and sterilized media.

This fact has also been realized and met by the use of a number of agents, among which the best known are probably blood, ascitic fluid, hydrocele fluid, pleural fluid, and potato. All of these have definite uses for which they cannot be easily replaced. But their use outside these specific instances has been limited. The four former substances cannot always be obtained, and in the case of the potato, special care must be employed to maintain sterility during processing.

In 1891, Van Slyke referred to the use of coconut milk as a bacteriologic culture medium. In 1897, Erwin F. Smith used coconut meat as a medium for yeast and molds. And in 1920, B. A. Linden utilized coconut juice or milk as

*From the Biltmore Hospital, Asheville.
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culture and by fractional sterilization made an agar medium. But I have been unable to find reference to the use of coconut milk in the unsterilized state as an uncooked nutrient substance which may be added to general culture media to enhance their nutrient properties.

In the following experiments, the juice of fresh, ripe coconuts was used; the ripe fruit was used rather than the green, because it is more readily obtainable. The coconuts were obtained as fresh as possible. The eyes were soaked in alcohol and then burned. A large aspirating needle was then inserted, stylet in place, in the softer eye (stylet withdrawn and sterile syringe attached), and the milk withdrawn. (The average coconut was found to contain 100 to 125 c.c. of slightly turbid fluid.) Smears were made from the centrifuged sediment of a portion of the fluid to determine the presence of possible organisms. The fluid was then added in the proportion of 10 to 25 per cent to broth or agar medium. The medium was then adjusted to the pH recommended for the organism under consideration, and the tubes inoculated in the manner required by the specific organism. Toward the end of the coconut season and before the fresh fruit comes to market, it was found well to incubate the milk overnight in a sterile corked container, to insure it against the presence of organisms.

The organisms used in this experiment were *Staphylococcus aureus, Streptococcus viridans, B. fecalis alcaligenes,* pneumococcus, gonococcus, meningococcus, *B. welchii,* and tubercle bacilli. In each instance, unless otherwise stated, the medium to which the coconut milk was added was either plain nutrient broth, plain agar, or both.

I. In the case of the *Staphylococcus aureus,* 2 per cent glucose agar and plain nutrient agar were used in comparing media with the coconut milk enriched media. After twelve hours of incubation, the colonies on the coconut milk media were half as large again as those on the glucose medium and slightly more numerous. The growth on the enriched agar was twice that on the plain agar.

II. In the case of the *Streptococcus viridans,* blood agar was used as a comparing medium. After twelve hours incubation, growth was nearly twice as abundant on the blood agar, but there was good growth on the coconut milk medium. This being true, the organism was replanted on 2 per cent glucose and again on the coconut milk medium. In this instance, the growth was one and one-half times greater on the latter. Smears were made from both these last cultures, and the organisms grown on glucose agar at twelve hours showed very short chains and often no chains at all; while those on the coconut milk agar showed longer and more characteristic chain formation. Although in this case the coconut milk medium is not superior to the blood agar, it was found to be superior to the glucose agar. This fact can be of value to office and small institutional laboratories where suitable blood is not always available. Comparative cultures were also made on enriched and plain nutrient broth. Growth was doubled in the enriched broth.

III. In the case of *B. fecalis alcaligenes,* plain agar and nutrient broth were used as comparing media. Growth after twelve hours was twice as great and the colonies more luxuriant on the coconut milk agar than on the plain. Broth culture was more turbid in the case of the coconut milk broth.

IV. Blood agar was used as a comparing medium for the study of the

pneumococcus. Again as in the *Streptococcus viridans*, the growth was only about half as great on the coconut milk agar as on the blood agar. However, smears from both cultures showed definite short chain and capsule formation. Again as in the case of the *Streptococcus viridans*, further comparison was made between plain agar and the coconut milk enriched agar. The colonies were larger and the growth nearly twice as great on the coconut agar. Smears from these two cultures showed chain and capsule formation as before on the coconut agar, and only a rare chain on the plain agar.

Swartz medium and plain agar were used as comparison for the study of the gonococcus. The reading in this case was made in seven days. Moisture was maintained and the oxygen tension kept as nearly 20 per cent as possible. Growth on the Swartz medium was approximately one-fourth greater than on the enriched plain agar, but there was quite a visible growth on the latter. This fact should again be of value to small laboratories where ascitic, pleuritic or hydrocele fluid is not always available. No growth was obtained on the plain agar culture.

Blood serum medium and plain agar were used for comparison for the meningococcus. In this instance, the growth appeared more rapidly on the coconut milk agar but was less luxuriant after growth was established. Growth was absent to scant on the agar cultures.

Plain broth and plain agar were used for comparison for the *B. welchii* concentrated beef broth. In the concentrated broth and the coconut broth growth was almost equal and in each instance luxuriant. Growth was much less luxuriant on plain agar and in plain beef broth.

Potato medium and plain agar were used for comparison in the work on tubercle bacilli. The coconut medium did not shorten the period of lag, and growth was about half as heavy on it as on the potato medium. There was no growth on the plain agar slants. In this instance, the medium to which the coconut milk was added was an egg yolk glycerin agar.

An analysis of coconut milk, reported by E. M. Caray in the *Philippine Agriculturist*, was obtained through the courtesy of the bacteriological section of the U. S. Food Division and is as follows:

Specific gravity at 15.5	1.023
Water at 60° C.	0.96 per cent
Ash	0.9 per cent
Acid constituents	48.0 per cent
Glucose	0.5 per cent
Cane sugars	2.6 per cent

The sugars present were found to be sucrose, fructose, and glucose; the per cent was given only for those mentioned in the above table.

The vitamin content of coconut milk made by J. H. Axmayer, also obtained through the U. S. Food Division, states that "Vitamin B is present in small amounts, while factor G (B_2) is present in appreciable quantities. Vitamins A and B have been reported in the meat."

SUMMARY

Sterile coconut milk is easily obtained by aseptically aspirating ripe fresh coconuts. The addition of this noncooked, nonsterilized nutrient agent nearly doubled the cultural qualities of ordinary nutrient broth and agar in the cases

of all organisms tested, and was found to be superior to the usual medium used in the case of *Staphylococcus aureus, B. fecalis alcaligenes,* and *B. welchii.* In the case of the meningococcus, it was found to be equally as good as the medium usually used. In the cases of the *Streptococcus viridans,* pneumococcus, gonococcus, and tubercle bacilli, it was found to be inferior to the preferred selected media.

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PREPARATION OF COLLOIDAL GOLD SOLUTION*

R. D. Scott, B.S., and Goldie De Long, Columbus, Ohio

ON SEARCHING the literature it appears the first recorded preparation of colloidal gold solution, or gold sol, was made by Faraday[1] in 1857. His method was the reduction of a solution of gold chloride by phosphorus dissolved in ether.

Bredig[2] in 1897 prepared gold sol, passing an arc between gold electrodes under water.

Zsigmondy[3] in 1898 presented the method of reducing gold chloride in solution made alkaline with potassium carbonate, using formaldehyde as a reducing agent. He also made the important observation that flocculation of gold particles with an electrolyte, such as sodium chloride, is prevented by definite protein concentrations, specific for each protein.

To Lange[4] is due credit for adapting this phenomenon to the examination of spinal fluids. He observed in 1912 that in suitable saline concentration those with syphilitic involvement cause flocculation of gold, while normal spinal fluids do not. He proposed the scale of dilutions, color ratings, and form of reporting now used in distinguishing the several types of syphilitic affections of the central nervous system.

Various modifications of Zsigmondy's preparation technique have been proposed by numerous investigators, their number attesting to the difficulty of preparing a satisfactory solution. Those most in use are of three general types: (1) the Eike[5] modification (1913), using glucose instead of formaldehyde; (2) the Miller[6] modification (1915), using oxalic acid together with formaldehyde; and (3) the Mellanby[7] a new method (1923), using potassium oxalate without formaldehyde.

The glucose method yields beautiful preparations which, however, do not seem so well adapted for clinical use as those prepared by the Miller procedure, since in our experience they are less sensitive. While we have not tried

*From the Ohio Health Department Laboratories, Columbus.
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out the oxalate procedure of Mellanby, it does not appear suitable for the preparation of large quantities.

A satisfactory colloidal gold solution may be defined as one which gives typical reactions with pathologic spinal fluids and no reaction with normal fluids, under the test conditions.

Satisfactory preparations will have the following characteristics:

The color by transmitted light is red, variously described as rose red, salmon red, or cherry red, with no trace of blue tint. By reflected light the color is brown, clear or with only slight turbidity.

The reaction lies between pH 6 and 7. Five volumes are precipitated by 1.7 volume of 1 per cent sodium chloride, largely within an hour; completely, overnight.

Usually, but not always, such solutions will be found satisfactory for clinical use.

Numerous factors influence the preparation of gold sol. There is lack of agreement both as to their relative importance and whether a given factor is favorable or the contrary. Thus, some observers believe that the distilled water used should be of the highest purity obtainable; others contend that traces of certain impurities are helpful, for example, those derived from rubber connections.

Some maintain that sunlight is necessary; others hold that its effect is not material. Some prescribe the addition of the several reagents separately and at definite temperature stages; some add them together.

The temperature of reduction is considered critical, but temperatures of 85°, 90°, 95°, and 100° C. are variously recommended.

Some workers use involved methods of adjusting the final reaction to neutrality; others consider it unnecessary or impractical to attempt any correction. Some prefer rapid heating, some slow. To date, over a period of four years, we have prepared 320 liters of satisfactory colloidal gold and some which were unsatisfactory. We have experienced considerable difficulties, but more in the past than at present.

We employ the Miller modification in so far as using both oxalic acid and formaldehyde as reducing agents.

The procedure of Miller and his co-workers is outlined: Heat 1000 c.c. of distilled water over a burner. At 60° C. add 10 c.c. of 1 per cent gold chloride and 7 c.c. of 2 per cent potassium carbonate. At 80° C. add 10 drops of 1 per cent oxalic acid. Remove from flame at 90° C. and add 5 c.c. of 1 per cent formaldehyde (40 per cent solution diluted 1 in 40), or enough to produce a pink color.

We found that double distilled water seemed to work better than triple, and following the installation of a new, very efficient still we were able to use single distilled water, letting the distillate come over warm and receiving it in a pyrex bottle.

It was found unnecessary to adhere to the prescribed temperatures; the gold chloride, potassium carbonate, and oxalic acid may be added successively, with stirring, at temperatures between 50° and 60° C. Heating is then continued to boiling instead of 90°, the beaker is then removed from the flame, and the formaldehyde is added. We believe that the reduction proceeds more

rapidly in good daylight and that it is promoted by the presence of a previously formed film of gold on the beaker wall, also by sufficient stirring.

In our opinion the most important factor which determines whether the solution is clear and satisfactory for the purpose of the test, or turns turbid and unusable, is the technique of the reduction with formaldehyde.

It was observed that the amount added, the time allowed for reaction, and the temperature are all of significance. Accordingly, we gradually evolved the procedure of adding a definite volume and depend on the time and temperature factors for completion of the reaction.

Our procedure, which we have employed for the past four months, by means of which over 100 liters of uniformly satisfactory sol have been prepared, is to add at once 3.0 c.c. of the formaldehyde solution, then to stir continuously. A pink color soon develops, gradually deepens, then suddenly flashes to the desired light cherry red color. This final color change usually develops as the temperature drops to from 85° to 80° C.

Immediately after preparation, the solution is transferred to pyrex bottles and is stored in the dark, under refrigeration. The beaker used is partially, but not completely, freed from deposited gold film by wiping with a clean towel, and after rinsing with distilled water is again ready for use. The strength, as well as the purity of the reagents used, must be considered. We have noted that while different lots of gold chloride, oxalic acid, and formaldehyde have not deviated materially from their theoretical contents, three brands of C.P. grade so-called anhydrous potassium carbonate ranged in alkaline strength from 82 to 89 per cent of theory. Thus, varying strengths of this reagent may also tend to explain the difficulties of preparation.

We have found through experience—not by calculation—that using potassium carbonate 83 per cent anhydrous strength, the optimum volume in relation to the other reagents used is 7.8 c.c. of 2 per cent solution.

It has been our experience, using the oxalic acid-formaldehyde method of reduction, that if the finished solution is clear, or nearly so, and is free from blue tint, it will almost invariably be found satisfactory in actual use.

We make no adjustment in reaction after preparation and determine whether a given solution is suitable for use by its behavior with known normal and paretic spinal fluids. If slightly insensitive, then in the next lot prepared we add 3.2 c.c. formaldehyde solution instead of 3.0 c.c.; if slightly oversensitive, we add 2.8 c.c. in the next preparation.

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A MODIFIED APPARATUS FOR MILKING SMALL LABORATORY ANIMALS*

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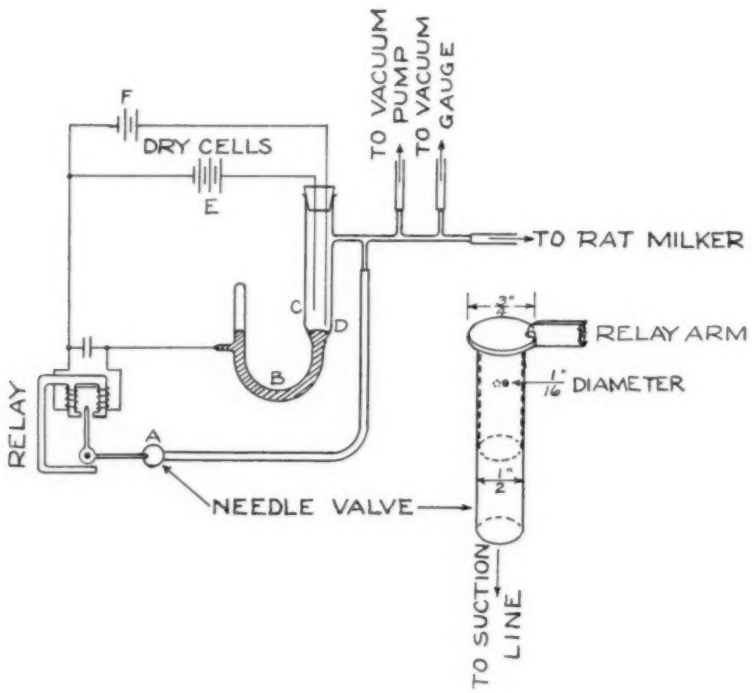
IN 1937, Cox and Mueller[1] described an apparatus suitable for milking small laboratory animals. The essential part of this report was the perfection of conditions necessary to obtain milk from rats. As an added convenience, an automatic device for obtaining pulsations in negative pressure (through the clamping action of a magnetic switch) was described. Temple and Kon[2]



confirmed the original findings and obtained pulsations in negative pressure through the operation of a piston.

The present note deals with a simplification of the early apparatus.[1] The magnetic switch (which operated with some uncertainty and much noise) has been replaced by a needle valve operated by the movable arm of an ordinary polarized telegraph relay. The entire apparatus (as indicated in Fig. 1) is readily constructed, and operates quietly and efficiently.

When suction is applied, the mercury rises in the U-tube, *B*, and completes the circuit at electrode, *D*. The current from the two dry cells, *F*, is insufficient

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to throw the relay and break the suction. When the mercury rises to electrode *C*, the effect of the three cells, *E*, throws the arm of the relay and opens the needle valve *A*, admitting air. When the contact at *C* is broken, the residual current (from *F*) is adequate to hold the needle valve (relay) in the "open" position. When, however, contact is broken at *D*, the arm of the relay, animated by the permanent magnet of the relay, closes the needle valve and the cycle begins again. This system of balanced electrical circuits gives the lag necessary in effective milking.

The needle valve is essentially two machined tubes with small holes in close approximation. The notched head, which fits into the arm of the relay, is attached to the inner tube, while the outer tube (connected to the suction line) is held firmly in place and is machined to fit tightly against the underside of the notched head. The two small holes are adjusted to open or close with the movement of the arm of the relay.

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A METHOD FOR THE DETERMINATION OF BROMINE IN A PROTEIN-FREE FILTRATE*

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THE recent interest in bromine metabolism and medication has brought an imperative need for an accurate and simple method for determining bromine in blood. The existing methods for the determination of bromides are too complicated and time-consuming for routine use in the hospital. The first step in nearly all of these procedures requires preliminary destruction of organic material. This is accomplished either by drying the sample and igniting it with an alkali[1] under carefully controlled temperature conditions, or by the wet oxidation[2] of the sample in acid. Whether the organic material is destroyed by the wet or dry method, the bromine is usually separated from the other halides by selective oxidation in an acid medium followed by distillation or aeration of the bromine or its oxidation products into a suitable absorption system.[1a-1d, 1f, 1j, 1k, 2a, 2c] The amount of bromine absorbed may be measured by the iodine liberated from added potassium iodide. The iodine may be determined either volumetrically or colorimetrically.

Another colorimetric procedure[1e] depends upon the production of a pink color (eosin) when an aqueous extract of the ashed sample, buffered with an acetic acid-sodium acetate solution, is treated with chloramine-T after addition of

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a drop of alkaline fluorescein solution. Color comparisons are made with test tube standards containing varying concentrations of bromine similarly treated.

In still another colorimetric procedure the bromide ions in an aqueous extract of the ashed filtrate are oxidized to bromine in the presence of decolorized fuchsin.[1g, 1h, 2e] This procedure gives rise to the development of a lavender or pink-colored compound which can be isolated on Fuller's earth and then redissolved in chloroform for use in a colorimeter (Denigès and Chelle reaction[3]).

Malamud[4] developed a colorimetric method for the determination of bromides based on the production of a brown color following the addition of a solution of gold chloride to a protein-free blood filtrate. This procedure greatly shortens the time required for a blood bromide determination and gives consistent results with blood drawn from patients receiving bromine medication (extremely high blood bromide content). However, the bloods of normal persons and patients with low blood bromide content, as determined by the method to be described, do not contain enough bromine to produce a satisfactory color with gold chloride.

Using a potentiometric method with a silver-silver bromide electrode, Hastings and van Dyke[5] titrated a protein-free filtrate with silver nitrate and obtained a curve from which the amount of each halide present could be determined. This method is cumbersome and requires the use of fairly expensive apparatus with which the ordinary laboratory technician is not usually familiar.

The method to be described in this paper consists of a modified Denigès and Chelle reaction applied directly to a protein-free filtrate. The oxidation of organic material is omitted since our experiments to be described later show this step to be unnecessary. A modification of the colorimetric procedure as originally described is the substitution of acetone for chloroform as a solvent for bromofuchsin derivative. The relative solubilities of the halogenated fuchsin derivatives are the same for acetone as for chloroform. However, the colors developed in an acetone solution are far better for colorimetric work than those developed in chloroform solution, and since acetone and water are miscible, there is less tendency for an acetone solution to be cloudy.

EXPERIMENTAL PROCEDURE

Reagents.—(1) Fuchsin reagent. A solution prepared by dissolving 0.05 gm. basic fuchsin in 500 c.c. warm water is cooled and treated with 5.0 c.c. concentrated hydrochloric acid. Five cubic centimeters saturated sodium bisulfite solution are then added, and the solution allowed to stand until decolorized (overnight). Such a solution is stable for three or four months at room temperature.

The sensitivity of this reagent to bromine may be increased by aerating the excess sulfur dioxide from the solution. However, the resulting solution is extremely unstable at room temperature and becomes colored within twelve hours, but if kept in a refrigerator this solution remains colorless for nearly a year. Nevertheless, the unaerated solution is much more practical for routine analyses.

(2) Bromine standards. (a) Stock solution—0.001 gm. bromine per c.c. prepared by dissolving 1.49 gm. potassium bromide in a liter of distilled water.

(b) Working standard—0.000001 gm. (1 gamma) per c.c. prepared daily by making appropriate dilutions of the stock solution.

(3) Concentrated sulfuric acid.

(4) Potassium persulfate.

(5) Saturated sodium bisulfite.

(6) Fuller's earth.

(7) Acetone.

Procedure.—A small amount of persulfuric acid is formed by adding 3 or 4 drops of concentrated sulfuric acid to 30 or 40 mg. potassium persulfate in a clean dry test tube and allowing them to react for three or four minutes. Ten cubic centimeters of a protein-free blood filtrate prepared according to the usual procedure of Folin and Wu are then added, followed by 1 c.c. fuchsin reagent. Within a few minutes after mixing the reagents by inverting the test tube, the solution becomes violet colored, due to the reaction of the bromine (formed by the oxidation of the bromide with persulfuric acid) and the fuchsin reagent. After this solution stands for a few minutes, the color changes to pink; this indicates that the oxidation of the bromine has been completed. The action of the persulfuric acid is then stopped by adding a drop of saturated sodium bisulfite solution. This treatment restores the violet color of the solution.

The halogenated compounds of fuchsin are absorbed on about 2.5 gm. Fuller's earth mixed well with the solution. After centrifugation, the supernatant liquid is discarded and the tube allowed to drain for two or three minutes. The bromofuchsin compound is then separated from the Fuller's earth by shaking well with 5 c.c. of acetone. After a second centrifugation, the pink-colored solution is decanted and compared in a colorimeter with a standard containing 10 gammas bromide solution treated in the same way.

When more than 5 mg. bromine per 100 c.c. are present (bloods from patients receiving bromide medication), the colored compound is not completely absorbed on the Fuller's earth. However, acetone (3 c.c.) added directly to the colored solution after the addition of the saturated bisulfite solution, dissolves the colloidal bromoderivative producing a color suitable for comparison with a standard treated in the same way.

Blood Bromine Content of 10 Students

Student No.	Br in mg. per 100 c.c. whole blood
1	0.71
2	1.57
3	0.92
4	0.65
5	0.64
6	0.64
7	1.12
8	0.89
9	1.00
10	0.62

DISCUSSION

The use of acetone as a solvent for the bromofuchsin compound produces a solution having a much deeper and more intense color than when chloroform is used. Small differences in bromine concentration are much easier to detect

colorimetrically. Furthermore, since acetone is soluble in water, temporary emulsions which form between chloroform and water are avoided. The relative solubilities of the halogenated fuchsin compounds are the same for chloroform as for acetone, the iodo and the bromo derivatives being soluble and the chloro derivative insoluble in acetone.

The mere presence of bromine in a protein-free blood filtrate does not preclude the existence of bromine in the protein material precipitated when preparing the filtrate. Therefore, this material was dried and examined separately for bromine. Three different procedures for the destruction of the organic material were as follows: (1) ashing the dried precipitated protein material at 450-500° C. in the presence of five times its weight of sodium carbonate, (2) wet oxidation with a chromic sulfuric acid mixture, and (3) wet oxidation with a chromic sulfuric phosphoric acid mixture. Solutions prepared in the first case by dissolving the ash in water, in the second by aerating, and in the third by distilling the bromine into an alkaline solution were then examined for the presence of bromine by our procedure. Negative results in every case established the absence of bromine in the precipitated protein material.

When the fuchsin reagent is treated with iodine, a reddish violet iodo compound soluble in acetone, chloroform, and alcohol is formed. The iodo fuchsin derivative cannot be separated from the bromo compound by our procedure. When less than 20 gammas of iodine are present in 10 c.c. of solution used for the determination, the color of a solution of the iodo derivative in the acetone is the same as that of the bromoderivative. Consequently, if iodine in amounts less than 20 gammas is present in the 10 c.c. of solution being analyzed for bromine, the color obtained by our procedure will be due to iodine as well as bromine, and the results will be too high. When more than 20 gammas of iodine are present, the acetone solution of the iodo fuchsin compound differs in color from an acetone solution of the bromo derivative. Solutions of bromides containing more than 20 gammas of iodine in 10 c.c. being tested cannot be compared with a bromine standard.

VALUES (IN GAMMAS) OBTAINED WHEN 10 C.C. OF SOLUTION ARE ANALYZED FOR BROMINE IN THE PRESENCE OF LESS THAN 20 GAMMAS IODINE

Gammas Bromine in Standard (10 c.c.)	Solutions to Be Tested (10 c.c.)		Apparent Gammas Bromine as Determined in 10 c.c.
	Conc. Bromine in Gammas	Conc. Iodine in Gammas	
10	5	5	10
10	3	7	10
10	7	3	10

The iodine content of normal blood varies from 4 to 6 gammas per 100 c.c. Since the bromine concentration of blood is at least a hundred times that of iodine, the blood iodine will not materially affect the results for the blood bromine unless the patient is receiving iodine medication.

The fuchsin reagent used in the bromine determination is similar to the well-known Schiff's aldehyde reagent, which when treated with aldehydes produces an immediate red color without the addition of persulfuric acid as an oxidizing agent. The absence of such reactions in the case of the protein-free blood filtrate eliminates the possibility of an increase in apparent bromine concentration due to the reaction of aldehydes with fuchsin. The constant values for blood bro-

mides in protein-free filtrates kept several hours at room temperature also establish this fact.

BROMINE CONTENT OF PROTEIN-FREE FILTRATES KEPT SEVERAL HOURS AT ROOM TEMPERATURE

Fresh Filtrate	1 hr.	2 hr.	4 hr.	6 hr.	15 hr.	24 hr.
0.71	----	----	0.76	----	0.81	----
1.57	1.53	----	----	1.54	1.42	----
0.92	----	0.94	0.85	----	0.87	----
0.65	0.70	----	0.68	----	0.70	0.71

SUMMARY

1. A colorimetric method for determination of bromine in a protein-free filtrate is described.

2. The procedure is relatively rapid and the method may be applied generally with the exception of patients receiving iodine medication.

3. It has been shown that the blood bromine is entirely in the protein-free filtrate and that the protein coagulum contains no bromine.

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PHOTOELECTRIC COLORIMETRY
II. QUANTITATIVE DETERMINATION OF BLOOD NONPROTEIN NITROGEN, UREA, CREATININE, CREATINE, GUANIDINE, AND AMINO ACIDS*

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INTRODUCTION

IN THE first paper of this series[1] is given a brief discussion of photoelectric colorimeters, and it is needless to repeat the same here. It is only necessary to add that we feel that the null-point, two-cell instruments are quite superior to the one-cell, direct-reading types, and, therefore, we have used this type of apparatus. A fairly detailed discussion of the instrument, together with a cut of the same, is given in Fig. 1 of paper I.† In the work outlined below we have not tried to introduce any actually new methods, but have only modified one of the more standard procedures for use with the electrical instrument. We have also given curves (for use with the instrument illustrated), and have pointed out the most satisfactory type of filter for each determination. Wratten (gelatin) light filters, mounted in glass, were used exclusively.

EXPERIMENTAL

Use of the Instrument.—The photoelectric colorimeter (Fig. 1, paper I) is turned on fifteen to twenty minutes before using to allow it to "heat-up," and thereby reach a constant output. The glass cell or cup‡ is then filled with water (or blank of the reagents) placed in the instrument, the milliamperage output of the photoelectric cell set at some definite value (0.40 Ma.), and the galvanometer adjusted to the neutral position with the conduction set at 100. Then the cup is filled with the solution to be tested and the per cent light transmission found, the output of the photoelectric cell being held at 0.40 Ma. all the while. From the graph for this determination, the concentration of the unknown is read off directly.

If filters are used, the current output of the photoelectric cell is too low to be read accurately; consequently the output is adjusted (at 0.40 Ma.) without the filter in place, and the incoming voltage noted. Then by means of a voltage regulator the incoming voltage is held at this value, while the filter is put in place, the instrument adjusted, and the unknown reading taken. The recommended procedures are given in more detail below.

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†Manufactured by Eimer and Amend, New York City.

‡These cells are standard glass absorption cells, 1 cm. thick (inside measurement), and hold approximately 18 c.c. of fluid. It is convenient to have at least two such cups, and to use one for the blank (or water) and the other for the unknown solution. The cups must be kept very clean by washing with soap and water after each determination.

NONPROTEIN NITROGEN

Principle.—This is essentially the method of Folin and Wu,[2] using a selenium digestion mixture.[3, 4] The tungstic acid blood filtrate is digested with acid and then nesslerized. From the value for the light transmission of the colored solution, the concentration of nonprotein nitrogen is obtained directly from a graph.

Procedure.—Prepare a protein-free blood filtrate by adding one volume of blood to seven volumes of water, shaking and waiting three to four minutes to allow the corpuscles to lake, and adding one volume of ⅔ normal sulfuric acid* and one volume of 10 per cent sodium tungstate. Shake and filter or centrifuge at once. The filtrate should be crystal clear.†

Measure 5 c.c. of this filtrate into a nonprotein nitrogen tube (pyrex test tube graduated at 35 and 50 c.c.), and add 1 c.c. of selenium digestion mixture.‡ By means of a microburner, heat the solution under a hood until white sulfur trioxide fumes begin to fill the tube. Then reduce the size of the flame greatly, and continue heating (never at any time allowing the flame to touch the tube above the level of the liquid) until the solution is clear. A slight amount of brown flakes in the top of the tube is of no harm, as this material floats on the surface of the liquid through the rest of the procedure. After cooling for a few minutes, dilute with water nearly to the 35 c.c. mark, and add 15 c.c. of diluted Nessler's solution.§ Dilute to 50 c.c. with water, mix, and read at once in the photoelectric colorimeter, after first setting the instrument with the cup filled with a blank of the reagents (35 c.c. of water + 15 c.c. of Nessler's) and using a No. 47 Wratten filter. A current of 0.40 Ma. is used (without the filter in place). A voltage regulator must be employed if the voltage is not constant. The nonprotein values are read directly from the graph in Fig. 1. If the value is above 60 to 80 mg., the analysis had best be repeated, using a smaller amount of the filtrate. If speed is necessary, such a sample may be saved by adding to the nesslerized unknown one or more equal parts of water (containing 15 c.c. of Nessler's + 1 c.c. of digestion mixture per 50 c.c. of volume), and redetermining the light transmission.

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*Prepared by adding 18.6 c.c. of concentrated sulfuric acid (94 per cent, sp.gr. 1.84) to water and making the volume up to 1000 c.c.

†Note that the reagents are added in the reverse order. This gives a more complete precipitation of the proteins, and the material can be filtered at once. This method of precipitation seems to be necessary in the determination of blood creatine.[5]

‡This is slightly different than either of the mixtures of Campbell and Hanna[3] or Reis and Powers.[4] It consists of 100 mg. of selenious acid (selenium dioxide) added to 200 c.c. of a 3:1 mixture of concentrated sulfuric and phosphoric acids. It is colorless and quite suitable for photoelectric work.

§The Nessler's solution is prepared from a mixture of the double iodide and water, the dilutions being different for urea and nonprotein nitrogen determinations.

Double Iodide. To 75 gm. of potassium iodide and 110 gm. of iodine in a 250 c.c. flask, add 50 c.c. of water and 70 to 75 gm. of metallic mercury. Shake the flask continuously and vigorously until the dissolved iodine has nearly all disappeared. The solution becomes quite hot. When the iodine color is visibly pale, cool in running water. Continue to shake until the red color of the iodine has been replaced by the green color of the double iodide. Do not cool too soon! Separate the solution from the excess mercury by decantation, and wash with liberal amounts of distilled water. Dilute the washings and solution to a volume of 2000 c.c. with water.

Nessler's Solution for Nonprotein Nitrogen. Dilute one part of the double iodide with one part of distilled water, and add four parts of 10 per cent sodium hydroxide (freshly prepared from C. P. chemicals). This solution keeps several months if stored in a brown glass-stoppered bottle.

UREA NITROGEN

Principle.—The blood filtrate is treated with a solution of urease and the color developed by direct nesslerization. This method is a modification of the method of Karr[6] similar to that outlined by the authors.[7]

Procedure.—Transfer 5 c.c. of tungstic acid blood filtrate into a test tube graduated at 25 c.c., and add 2 drops of glycerin urease solution* and 4 drops of phosphate buffer solution.† Incubate in a water bath at 45° to 50° C. for fifteen minutes. Cool, dilute up to about 20 c.c. with water, add 3 c.c. of urea Nessler's solution,‡ and dilute to 25 c.c. Mix and find the light transmission

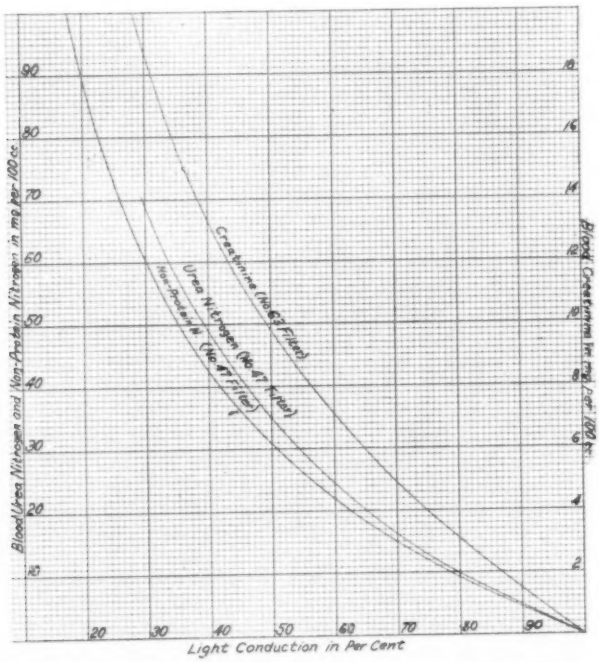

Fig. 1.—Photoelectric light transmission curves for nonprotein nitrogen, urea nitrogen, and creatinine. The type of filter is specified on the curve.

in the photoelectric colorimeter as with the nonprotein nitrogen, after first setting the instrument at 100 with the cup filled with water, Nessler's solution, buffer, and urease (made up to 25 c.c. in volume). A No. 47 Wratten

**Glycerol Urease Solution.* Shake 15 gm. of permutit with 200 c.c. of 2 per cent acetic acid, decant the aqueous phase, and wash two or three times with distilled water. To the damp permutit, add 50 c.c. of 0.001 normal sulfuric acid and 30 gm. of jack bean meal (Arlington Chemical Co.). Shake gently for thirty minutes, add 150 c.c. of glycerin, and mix well. Let stand overnight and filter through several thicknesses of gauze. Centrifuge the filtrate until essentially clear (there is an opalescent haziness that cannot be entirely removed). The filtrate is, however, very active and stable. The equivalent of 0.03 to 0.05 c.c. will convert 2 mg. of urea nitrogen completely into ammonia (in a 5 c.c. volume) at 45° to 50° C. in fifteen minutes. Two drops are sufficient for most urea determinations. Such an extract will keep its full activity for at least a year at room temperature and exposed to the ordinary room illumination; however, keeping the extract in the refrigerator is to be preferred if this is convenient. See Koch.[8]

†Phosphate Buffer. Dissolve 14 gm. of sodium pyrophosphate (U.S.P.) in water, add 2.6 c.c. of glacial phosphoric acid (85 per cent), and make the volume up to 250 c.c. with water.

‡Nessler's Solution for Urea. Mix three parts of the double iodide (see footnote § p. 433) with three parts of distilled water. To this mixture add 14 parts of 5 per cent sodium hydroxide (freshly prepared). The solution is best prepared fresh from the double iodide at least every three months.

filter is used, and a photoelectric current of 0.40 Ma. (with the filter removed). The urea nitrogen value is obtained directly from the curve in Fig. 1.

For values above 60 mg. per 100 c.c., the determination is best repeated using smaller amounts of filtrate; if necessary the colored solution may be diluted with one or more volumes of water (containing Nessler's solution, urease, and buffer), and the color transmission redetermined.

PREFORMED CREATININE

Principle.—This is essentially the method of Folin and Wu,[2] which is very widely used. The red color is developed by treating the blood filtrate with alkaline picrate, and the amount of color determined by measuring the light conduction.

Procedure.—To one volume (20 c.c.) of tungstic acid blood filtrate in a 50 c.c. flask, add one-half this amount (10 c.c.) of alkaline picrate solution,* mix, allow to stand eight to ten minutes, and read the light transmission in a photoelectric colorimeter (using a No. 63 Wratten filter and a current of 0.40 Ma., without the filter). The instrument is previously balanced at 100 with the cup filled with water and the reagent (in the ratio of 2:1). The creatinine is obtained directly from the graph in Fig. 1. For values above 20 mg., the analysis should be repeated with one-half the amount of filtrate (diluted with water); in case of a necessity the colored solution may be diluted with an equal amount of water and picrate, and the light transmission redetermined.

TOTAL CREATININE AND CREATINE†

Principle.—Creatinine is determined before and after autoclaving with hydrochloric acid (to convert the creatine into creatinine), and the creatine is calculated from the difference.

Procedure for Total Creatinine.—Precipitate the blood proteins in the manner given for nonprotein nitrogen.‡ To 15 c.c. of the filtrate in a 25 c.c. volumetric flask, add 0.3 c.c. of concentrated hydrochloric acid, and autoclave the flask for thirty minutes at 120° C. (20 pounds pressure). After cooling, neutralize with 10 per cent sodium hydroxide (using litmus paper as an indicator), and make the volume up to 25 c.c. After mixing, determine the creatinine on 20 c.c. of this solution, using the method for preformed creatinine. The value obtained from the graph (Fig. 1) is multiplied by 1.67 to give the total creatinine per 100 c.c. of blood.

Calculation of Creatine.—Subtract the preformed from the total creatinine, and multiply this figure by 1.16.

BLOOD GUANIDINE§

Principle.—The guanidines are extracted from blood by blood charcoal, autoclaved to convert creatine into creatinine, and a reddish color developed with a modified Tieg's reagent.

**Alkaline Picrate Solution.* Prepare by adding 5 c.c. of 10 per cent sodium hydroxide to 25 c.c. of 1.2 per cent (saturated) picric acid solution. This solution must be prepared fresh for each determination.

†Essentially the method of Folin and Wu[2] as modified by Andes.[5]

‡It is desirable to add the acid and tungstate in the reverse order to that advised by Folin and Wu, as the resultant filtrate usually remains entirely clear when autoclaved.

§Method of Andes and Myers.[9]

Procedure.—The method for preparing the extract given in the original paper was used unchanged. Since the procedure is fairly long, it is not repeated here. To the final extract, add 15 c.c. of water (instead of the 2 c.c. in the original paper) and 5 c.c. of the color reagent,* and centrifuge three to four minutes to remove the precipitate that always forms at this juncture. Adjust the colorimeter with a blank of water and reagent in the cup, using a No. 63 filter and 0.40 Ma. of current from the photoelectric cell (without the filter in place). Now fill the cup with the unknown solution, and read the light transmission within nine minutes from the time the color reagent was added. The guanidine is read directly from the graph in Fig. 2.

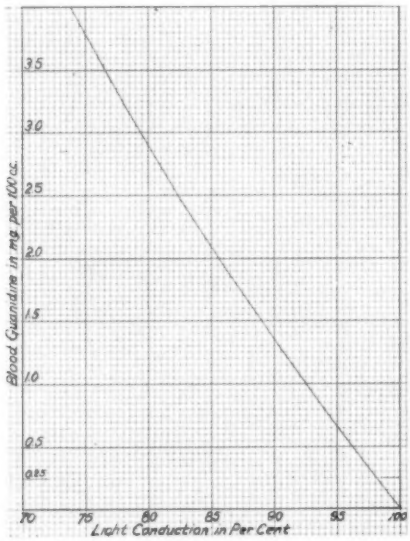

Fig. 2.—Photoelectric light transmission curve for guanidine (No. 63 Wratten filter).

BLOOD AMINO ACID NITROGEN†

Principle.—Blood of plasma amino acid nitrogen is determined directly on the protein-free filtrate, using a suitable color reagent.

Procedure.—To exactly 10 c.c. of tungstic acid blood (or plasma) filtrate in a 25 c.c. volumetric flask, add 2 c.c. of 1.5 per cent borax solution and 2 c.c. of a *freshly prepared* 0.5 per cent solution of sodium beta-naphthaquinone sulfonate (Eastman Kodak Co.). At the same time prepare a blank of the reagents, using 10 c.c. of distilled water instead of the filtrate. Let both flasks stand in the dark for eighteen to twenty-four hours. Now add to both flasks 2 c.c. of acid formaldehyde reagent,‡ and 2 c.c. of 0.1 molar sodium thiosulfate solution (not standardized).§ Dilute to 25 c.c. and let stand four to five minutes to allow bleaching of the excess reagent.

*Details for preparing the reagents are given in the original paper.[9] The color reagent should be made up fresh every day for most accurate work.

†Method of Danielson[10] slightly modified.

‡*Acid Formaldehyde Reagent.* Mix three volumes of 1.5 normal hydrochloric acid, one volume of glacial acetic acid, and four volumes of 0.15 molar formaldehyde (11.3 c.c. of 40 per cent formaldehyde diluted to 1000 c.c. with water gives a 0.15 molar solution of formaldehyde).

§*0.1 Molar Sodium Thiosulfate.* Dissolve approximately 25 gm. of pure crystalline sodium thiosulfate in water, and make the volume up to 1000 c.c.

Adjust the colorimeter (no filter) at 100 with the blank solution in the cup, with a photoelectric current of 0.40 Ma. Then fill the cup with the unknown solution, and find the light transmission. From the curve in Fig. 3 the concentration of amino acid nitrogen is found directly. For values above 20 mg. the determination is repeated with a smaller amount of the filtrate. Such high values are only rarely observed, and then only in cases of very severe liver injury.

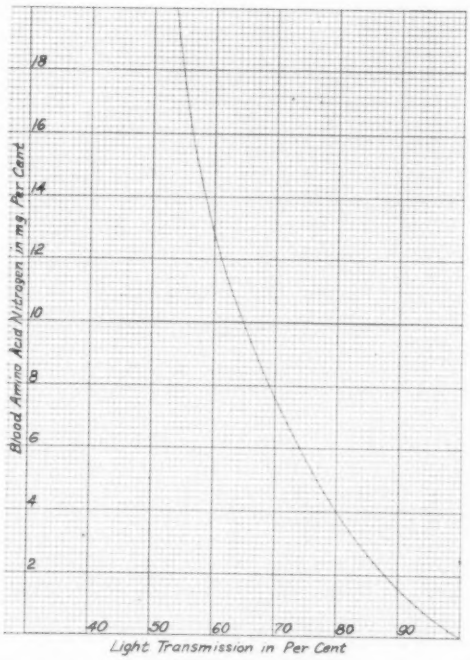

Fig. 3.—Photoelectric light transmission curve for amino acids (no filter).

DISCUSSION

In general, little need be said except to extol the additional accuracy and rapidity with which the photoelectric determinations can be carried out. After one determination alone, one is impressed with both of these points. The lack of necessity for preparing a standard solution is a marked advantage, especially with abnormal blood samples and also with standards that keep poorly. The freedom from eyestrain is a very welcome entity. The main objection is the first cost of the instrument.

The curves for urea, nonprotein nitrogen, and creatinine are given only for the lower part of the pathologic range. With the first two substances it is obvious from the graphs (Fig. 1) that values above 60 mg. are out of the more accurate part of the curve; hence we have not included them in preparing the graphs. The value so rarely exceeds 20 mg. for creatinine that it seemed advisable to plot the lower values on a larger scale than to include the entire range. The amino acid curve includes all values except the extremely high ones observed in severe hepatic destruction. In all the determinations the agreement between duplicate determinations is usually quite close.

The most important point (as was also discussed in the first paper of this series) is whether or not these curves can be used by another operator with another (similar) instrument, using other filters. While the best plan is for each worker to construct his own curve, this may not be necessary in routine work. The filters (especially if kept in the dark or in their cases) do not appreciably fade in one year's time. The cups are standard 1 cm. spectroscope cups, and are constructed very accurately. Since the photoelectric determination is made by the null-point method with a constant current coming from the first cell, we doubt if a different colorimeter will introduce any appreciable error. In fact, we believe that if the values do not need to be more accurate than 5 per cent, the curves listed may be safely used per se.

SUMMARY

1. Procedures for determining nonprotein nitrogen, urea, creatinine, creatine, guanidine, and amino acid nitrogen in the blood have been adapted for use in the photoelectric colorimeter.

2. Curves are given to facilitate the use of these methods, and it is believed that the curves may be used by other workers using similar instruments for regular clinical work.

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A SIMPLE RAPID METHOD FOR THE DETERMINATION OF TOTAL PROTEIN AND ALBUMIN CONCENTRATIONS IN BLOOD PLASMA, SERUM, OR OTHER BODY FLUIDS*

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THE concentrations of plasma total protein and albumin can be determined simply and quickly by centrifugalization after precipitation by a modified Folin-Wu technique. The results so obtained are more than adequate for clinical purposes, and can be readily carried out by the doctor himself or his office nurse. These methods were developed in response to the needs for methods simplifying the office management of Bright's disease, but can be applied as well to other body fluids and to plasma or serum in other diseases in which knowledge of protein concentration is necessary.

Of fundamental importance in these methods is the use of a centrifuge which runs at a constant speed (3,500 r.p.m.) for a constant time, regardless of voltage fluctuations, and with minimized temperature rise (due to "windage"). Such a centrifuge has been constructed and is now on the market. Furthermore, centrifuge tubes molded of transparent unbreakable plastic material are now available; these tubes are not only graduated identically and accurately, but also have flat bottoms so that the radial distance, and therefore the force of centrifugalization, is always constant. The methods to be described have all been carried out with the above equipment.†

METHODS

Principle.—One reads the volume of protein precipitated from 1 c.c. of plasma (or other body fluid) after the addition of sulfuric acid and sodium tungstate and after centrifugalization. Under certain standard conditions, 30 mg. of protein yield 1 c.c. of packed precipitate, so that the volume of precipitate multiplied by 3 gives the protein concentration directly in per cent.

Reagents.—1/10 N sulfuric acid; 10 per cent sodium tungstate solution; and saturated ammonium sulfate solution.

TOTAL PROTEIN

Procedure.—Place 1 c.c. of plasma into an accurately graduated 15 c.c. centrifuge tube. Add 8 c.c. of 1/10 N sulfuric acid; mix by inversion two or three times. Add 1 c.c. of 10 per cent sodium tungstate solution, and mix by inversion twice. Centrifuge as soon as possible at 3,500 r.p.m. for exactly five minutes. The flat-topped precipitate packs cleanly, and its volume is easily read.

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†The centrifuge and tubes are supplied by the Electric Manufacturing Co., 34 Dore St., San Francisco.

Calculation.—The volume of packed protein precipitate in cubic centimeters multiplied by 3 gives the total protein concentration directly in per cent.

Rarely the volume of precipitate is 2.5 c.c. or more (7.5 per cent or more); when this occurs, the determination should be repeated, using 0.5 c.c. of plasma,

TABLE I

PLASMA PROTEIN CONCENTRATIONS IN NORMAL INDIVIDUALS, GRAMS PER 100 C.C.

	TOTAL	ALBUMIN	GLOBULINS
	Per cent	*Per cent*	*Per cent*
	8.1	3.6	4.5
	7.2	4.5	2.7
	7.2	3.9	3.3
	6.9	5.4	1.5
	6.9	4.6	2.3
	6.9	4.6	2.3
	6.9	4.6	2.3
	6.9	4.5	2.4
	6.9	4.2	2.7
	6.9	3.9	3.0
	6.8	4.7	2.1
	6.6	4.1	2.5
	6.5	4.7	1.8
	6.5	4.6	1.9
	6.5	4.2	2.3
	6.3	4.5	1.8
	6.3	4.2	2.1
	6.3	3.9	2.4
	6.0	4.8	1.2
	6.0	4.5	1.5
Means	6.7	4.4	2.3
Means (Peters and Van Slyke[2])	7.0	4.4	2.6

TABLE II

PLASMA PROTEIN CONCENTRATIONS IN CERTAIN PATIENTS, GRAMS PER 100 C.C.

	TOTAL	ALBUMIN	GLOBULINS	DIAGNOSIS
	Per cent	*Per cent*	*Per cent*	
	6.0	2.6	3.4	Renal edema
	6.0	2.1	3.9	Renal edema
	5.6	2.1	3.5	Renal edema
	5.6	1.8	3.8	Renal edema
	5.3	2.0	3.3	Renal edema
	5.1	2.7	2.4	Renal edema
	4.8	3.1	1.7	Renal edema
	4.7	3.6	1.1	Renal edema (marked lipemia)
Means	5.4	2.5	2.9	Renal edema
	6.9	4.2	2.7	Cardiac edema
	6.9	3.6	3.3	Cardiac edema
	6.2	3.3	2.9	Cardiac edema
	5.9	3.0	2.9	Cardiac edema
	5.7	3.6	2.1	Cardiac edema
Means	6.3	3.5	2.8	Cardiac edema
	9.0	1.8	7.2	Multiple myeloma

8.5 c.c. of 1/10 N sulfuric acid, and 1 c.c. of 10 per cent sodium tungstate. The packed volume of precipitate should then be multiplied by 6.

ALBUMIN

Separation.—To 2 c.c. of plasma, add 2 c.c. of saturated ammonium sulfate solution, and mix by several inversions. Centrifugalization at 3,500 r.p.m. for fifteen minutes gives a clear or nearly clear supernatant fluid. *Complete* clear-

ing is more easily obtained by centrifugalization of two tubes, each with 2 c.c. of the plasma-ammonium sulfate mixture, or better yet, of four tubes each with 1 c.c. During this step, it is essential to allow the centrifuge to continue revolving freely after the current shuts off, for even gentle braking may cause turbidity in the albumin fraction. (At all other steps in these methods, the machine may be braked if desired.) The usual minor degrees of cloudiness have no appreciable effect on the determination of albumin, nor does the milky fluid of lipemic plasma.

Procedure.—The supernatant fluid is drawn off by means of a capillary pipette. Place 2 c.c. (equivalent to 1 c.c. of the original plasma) in an accurately graduated centrifuge tube, and add 7 c.c. of 1/10 N sulfuric acid; a precipitate forms, but immediately disappears on mixing by inversion two or three times.

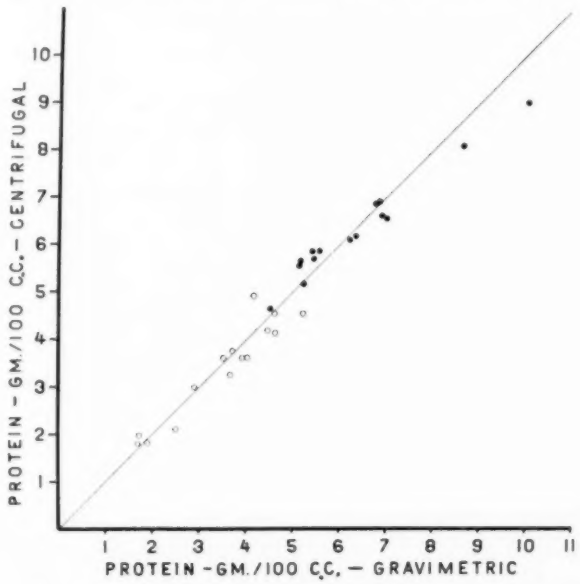

Fig. 1.—Comparison of results by the new methods with determinations by a standard gravimetric method. The line indicates perfect correlation. The dots indicate total protein, the circles albumin concentrations.

Add 1 c.c. of 10 per cent sodium tungstate, and invert twice. Centrifuge as soon as possible for exactly five minutes at 3,500 r.p.m. The precipitate of albumin packs cleanly.

Calculation.—The volume of packed precipitate in cubic centimeters multiplied by 3 gives the albumin concentration directly in per cent.

RESULTS

The concentrations of total protein and albumin by the simple methods given above have been checked against results obtained by a standard gravimetric method[1] on the same samples of plasma (2 mg. of potassium oxalate per c.c. of blood) and albumin solution (fifteen samples each). Fig. 1 gives the comparison of results by the two methods.

The average error was ±0.3 gm. per 100 c.c. Thirteen determinations were less than 0.25 gm. in error (of these, 9 erred less than 0.1 gm.), and 13 more were

within 0.5 gm. of the correct concentration. Three other determinations (on normal or high concentrations) deviated by 0.6, 0.6, and 0.7 gm. The thirtieth determination, while 9.0 instead of 10.1 gm./100 c.c., was done only routinely in the course of standardizing these methods, and led to the diagnosis of a previously unsuspected multiple myeloma, later confirmed by x-ray studies made because of the high total protein and low albumin concentration determinations.

In addition to the gravimetric standardization cited above, these methods have been used on 20 determinations of total plasma protein and albumin concentrations in normal individuals; 8 in patients with edema due to chronic glomerular nephritis, and 5 in patients with edema because of congestive heart failure. The results are given in the tables, and are those well known to be typical of these clinical conditions. The normal averages agree very well with those given by Peters and Van Slyke.[2]

OTHER BODY FLUIDS

These methods have been applied to an exudate (tuberculous pleural effusion), with results of 5.5 per cent for total protein (5.3 per cent by the gravimetric method) and 3.3 per cent for albumin concentration. Transudates (ascites with portal cirrhosis) have also been satisfactory, giving concentrations of 1.5 per cent, 1.2 per cent, and 0.9 per cent for total protein with 0.6 per cent, 0.45 per cent, and 0.45 per cent for the respective albumin fractions. (The first ascitic fluid was checked against the gravimetric method, which gave concentrations of 1.2 per cent and 0.6 per cent for total protein and albumin.) Preliminary work has shown that urinary and spinal fluid protein concentrations may also be determined by modifications of these methods.

DISCUSSION

The chief new features of the above methods are (a) the use of the Folin-Wu reagents, which give a more voluminous and more accurately readable precipitate than do other protein precipitants, and (b) the use of equipment which results in great constancy of centrifugalization. By their combined use, the methods become simple and are quickly carried out, and all but the most simple calculations are eliminated.

All of the above determinations have been made at a room temperature between 20° and 25° C. In this range, temperature does not appreciably alter the volume of packed precipitate from a given amount of protein. With warmer temperatures, the packed precipitate is less, and with colder temperatures greater, than when working at 20° to 25° C. Enough data have been obtained to permit the use of approximate correction factors. At 10° C. the observed protein concentration will be about 115 per cent of the correct concentration; at 15° C., 110 per cent; at 30° C., 90 per cent; at 35° C., 85 per cent. The methods are, of course, more accurate if carried out in rooms at 20° to 25° C. Specimens taken from an icebox should first be permitted to reach room temperature.

Great care should be taken to mix thoroughly the plasma and sulfuric acid before adding the tungstate, or the results may be too low; during the two or three inversions one must allow the contents of the conical tip to drain

well. The sodium tungstate solution should be reasonably fresh and neither too acid nor too alkaline.[3]

SUMMARY

Simple, quick, and clinically adequate methods have been described for the determination of total protein or albumin concentration in plasma, serum, or other body fluids. They may be carried out in the doctor's office by his nurse or by himself.

Thanks are due to Evalyn Barrett, B.S., for the gravimetric determinations of protein and albumin concentrations.

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DEPARTMENT OF REVIEWS AND ABSTRACTS

ROBERT A. KILDUFFE, M.D., ABSTRACT EDITOR

PNEUMOCOCCI, Microscopic Precipitin Test for Typing, Schaub, I. G., and Reid, R. D. J. A. M. A. 111: 1285, 1938.

Preparation of the Antigen.—Cultures of pneumococci from blood, spinal fluid, or other sources taken during life or at autopsy may be grown in meat infusion broth containing a few drops of serum or ascitic fluid, incubated at 37° C. for from six to eighteen hours. The culture is lyzed by the addition of two drops of a 10 per cent aqueous solution of sodium desoxycholate (containing 1:50,000 merthiolate to prevent the growth of molds) per cubic centimeter of culture. In from fifteen to thirty minutes the organisms are dissolved, and tests have shown that the lysate is sterile. The antigen is now ready for use. In some instances an excess of sodium desoxycholate in the presence of serum will cause the culture medium to gel. This may be liquefied by warming slightly without decreasing the efficiency of the test.

Preparation of the Typing Serum.—For making this test, horse antipneumococcus serum such as is used for macroscopic agglutination tests, diluted 1:10 in physiologic solution of sodium chloride, is used. Such dilutions have been found satisfactory after refrigeration for as long as six weeks.

Rabbit antipneumococcus serum as prepared for the Quellung test, diluted 1:1 in salt solution, has been used, but the reaction takes place more slowly, sometimes requiring from one to two hours for marked precipitation to take place. The reaction with the diluted horse serum takes place rapidly, and readings can be made after from fifteen to thirty minutes. By adding 1 per cent aqueous methylene blue to the horse serum the reading of results is facilitated, although the dye is not essential to the test. Dye is already present in the rabbit Quellung serum.

Attempts were made to place the organisms into groups by means of commercially prepared Quellung grouping sera or by mixing the typing sera prepared from horses in proportions based on the titer of the species antiserum, but results obtained were not sufficiently uniform to be considered satisfactory.

Technique of the Test.—The Petri dish-hanging drop technique described by Brown is used for making the test. A disk of moist filter paper is placed in the lid of the dish. The bottom of the dish is ruled on the bottom surface with a wax or diamond pencil, providing sixteen 12 mm. squares. In the performance of the test two Petri dishes are required for each organism to be typed. In one dish the antigen is set up against each of the fifteen types of antiserum (diluted), the sixteenth square being used for an antigen-salt control. In the second dish the remaining type antisera are set up. It has been found most practicable to add one platinum loopful of the antigen to each of five squares and mix them with an equal amount of the appropriate typing sera, and then to proceed with the next five squares in the same manner. This prevents drying of the loopful of antigen before it is possible to add the antiserum, as is likely to happen if the antigen is placed in all fifteen squares before the antisera are added. A 2 mm. loop of platinum rather than one of a platinum substitute is recommended. The loop should be thoroughly flamed and cooled each time before being introduced into the serum.

Reading of Results.—The mixtures of serum and antigen are held from fifteen to thirty minutes, and readings are made with the 16 mm. objective of the microscope, the light from the condenser being allowed to pass through the moist filter paper. Gentle rotation of the plates hastens the formation of large clumps of precipitate. The appearance of large clumps of precipitate indicates a positive reaction. Negative results are indicated by the absence of such clumping. Doubtful or questionable results are seldom encountered but can be eliminated by comparison with the control and a known positive. In using this technique

for the first few times it is suggested that known positives and negatives be set up as controls to familiarize the worker with typical reactions.

Infrequently, though occasionally, cross reactions occur. These may be distinguished from typical positive reactions with a little experience. Such a doubtful cross reaction is shown in B.

There appears to be no advantage in incubation or refrigeration of the mixtures.

SEDIMENTATION RATE, Normal, in Open Pulmonary Tuberculosis, Banyai, A. L., and Caldwell, E. Am. Rev. Tubere. 38: 491, 1938.

A study of the relationship between positive sputum and the sedimentation test in 5,274 cases of pulmonary tuberculosis is reported. It was found that in 2.1 per cent of these patients normal sedimentation rate and positive sputum were present at the same time. The symptomatology and simultaneous roentgenologic findings corroborated the presence of an active parenchymal pulmonary tuberculosis in the great majority of instances.

The analysis of the clinical material revealed, not only the possible coexistence of an active pulmonary tuberculosis and normal sedimentation velocity, but also that single or multiple cavities may coexist with a normal sedimentation rate.

The most plausible explanation of the varying rates of the sedimentation of the red blood cells is a disturbance of the colloidal balance of the blood. As a rule, there is a parallelism between the activity of the tuberculous process and the colloidal balance of the blood. Consequently, tissue destruction or tuberculous toxemia that upsets the equilibrium of the serum proteins is usually recognizable by an increased sedimentation speed.

The exceptions to this rule, as found by the authors and by others, can be explained by the hypothesis that there are certain cases of pulmonary tuberculosis in which the colloidal balance may remain normal during the active stage of the disease, or it may return to normal prior to cessation of activity of the tuberculous process.

The authors are of the opinion that, although these exceptions do not invalidate the value of the sedimentation test, its limitations should be recognized and, therefore, a normal sedimentation rate should not be considered an infallible evidence of the absence of an active pulmonary tuberculosis.

SYPHILIS, Frequency With Which Syphilitic Lesions Are Encountered in Postmortem Examinations, Bell, E. T. Arch. Path. 26: 882, 1938.

In a survey of 27,872 post-mortem examinations it was found that definite syphilitic lesions were noted in 2.77 per cent, and in approximately 2.5 per cent syphilis was shown to be the major cause of death. About 3.38 per cent of stillborn infants were syphilitic, and congenital syphilis caused about 2.63 per cent of the deaths in infants under 1 year of age. Congenital syphilis was a rare cause of death in infants more than 1 year of age.

Syphilis was an infrequent cause of death in persons dying in the second and third decades of life. The maximal mortality from this cause occurred in persons in the fourth, fifth, and sixth decades of life.

Acquired syphilis, in its lethal forms at least, was about twice as frequent in males as in females.

The major forms of lethal syphilis were syphilitic aortitis and neurosyphilis. The age distribution of these two forms was about the same.

TUBERCLE BACILLI, A Medium for the Culture, Isolation and Dissociation of, Steenken, W., Jr., and Smith, M. M. Am. Rev. Tubere. 38: 514, 1938.

A modification of Hohn's medium has been recommended. It differs from Hohn's in that it does not contain alanine; it contains only half the amount of asparagin; lacmoid is substituted for malachite green to impart a dark blue background instead of the light green produced by malachite green; it has an adjusted final pH of 6.2 as contrasted to Hohn's, which is unadjusted but usually has a final pH between 7.1 and 7.3.

It has also been shown that the modified medium is preferable to Corper's medium for colony study, since it eliminates marked atypical structures.

The use of the Fisher culture tube No. 14-927 is recommended for colony study.

The formula of the medium follows:

Distilled water	500	c.c.
Sodium phosphate	1.5	gm.
Potassium dihydrogen phosphate	2.0	gm.
Magnesium sulfate	0.3	gm.
Magnesium citrate	1.25	gm.
Asparagin	1.50	gm.
Glycerin	60	c.c.

Preparation of the Medium.—The salts are dissolved in 500 c.c. of distilled water at a temperature of about 80° C. The glycerin is added last and the mixture is sterilized in the autoclave at 10 pounds pressure for twenty minutes. This is the stock synthetic solution. To every 50 c.c. of synthetic stock used, 4.4 c.c. of a 1.0 per cent laemoid (this is made up in a 50 per cent alcoholic solution) solution is then added; 163 c.c. of whole egg mass is added, making a proportion of synthetic dye egg mixture of 1:3 and the final concentration of laemoid 1:5,000. This mixture is filtered through sterile gauze and the filtrate adjusted with N/1 hydrochloric acid, preferably with the aid of a potentiometer, to pH 6.0. The filtrate must be shaken thoroughly while adding the acid. It is now placed in tubes and inspissated for one and a half hours at 85° C. on two successive days. (The pH of the medium after inspissation becomes 6.2.) The medium may turn yellow on inspissation, especially in the deeper portions. This change in color is temporary, for on cooling, the medium assumes its original blue color.

It has been the desire of most originators of media for the cultivation and isolation of tubercle bacilli to add a dye bacteriostatic for all microorganisms other than the tubercle bacillus. It has been the authors' experience that practically all dyes incorporated in media to prevent the growth of secondary microorganisms have some inhibitory effect upon the growth of the tubercle bacillus. This inhibition is most pronounced when there are but few tubercle bacilli present in the material to be cultured.

It has also been observed that the digestion of the specimen is the most important factor in obtaining uncontaminated cultures. Careful digestion means vigorous shaking of the specimen with the proper concentration of sodium hydroxide or sulfuric acid until it is homogeneous and quite fluid, then incubation for one hour at 37.5° C.

It may be necessary to shake some specimens occasionally during this period of incubation to insure breaking up of clumps and allowing the digestion fluid to penetrate all of the material. Whatever time is spent in the preparation of the specimen for culture is well compensated for in the number of uncontaminated cultures obtained, regardless of the dye incorporated in the medium.

STREPTOCOCCI, Hemolytic, Precipitin Test Applied to Melbourne, Butler, H. M. M. J. Australia **11:** 501, 1938.

The results are reported of an examination of 113 strains of hemolytic streptococci isolated in Melbourne from human sources. Both group precipitin and biochemical reactions were studied.

With Lancefield's technique for the group precipitin test 25 strains could not be allotted with certainty to any one group, since 7 failed to react with any of the group sera, and 18 gave pronounced cross reactions. When formamide extracts were prepared from these strains according to the method of Fuller, all but one could be allotted to one or other serologic group. It had been noted, however, when the Lancefield tests were being carried out, that most of those strains of hydrochloric acid extracts which reacted with group A antiserum and also with one or more of the other sera differed from the strains which had reacted with the group A serum alone, in that they grew on 40 per cent ox bile blood agar plates and that in the primary cultures on blood agar plates the colony form was different. Further,

these atypical strains were not associated with signs of serious infection. In this paper those strains which belonged to group A, according to the formamide method, but which gave cross reactions with Lancefield's method, and which in addition were atypical either as regards tolerance for bile or colony form, have been referred to as atypical members of group A.

Of the 113 strains, 70 were typical members of group A, 15 were classed as atypical group A strains, 10 belonged to group B, 9 to group C, and 8 to group G; one failed to react with any of the four groups of antisera used.

Although the majority of strains associated with severe infections were typical members of group A, a group B and a group C strain, respectively, were the only organisms cultivated from the blood of two patients who were gravely ill. Of 46 strains associated with severe infections, 42 were typical members of group A; of 37 strains from patients with moderately severe infections, 24 were typical members of group A, while 30 mild cases yielded only four such strains.

The clinical condition of the patients with puerperal fever, cultures from whose vaginal swabbings yielded atypical group A streptococci, differed from that of the patients infected post partum with typical group A strains.

SILICOSIS, Study of, Matz, P. B. Am. J. M. Sc. **196**: 548, 1938.

Silicosis is the chief occupational disease in this country. The seriousness of silicosis is due to the fact that it is conducive to the inception of pulmonary tuberculosis. Tuberculous silicotics are a source of danger to their fellow workers as well as to members of their families, because of the ease with which tuberculous infection is transmitted. The disease results in a great economic loss to society.

A study of the classification of silicotic disease in a group of 167 veterans showed that 30 per cent were classified as first, second, or third degree silicosis; 11 per cent were cases of silicosis with nontuberculous infection; 42 per cent were cases of silicosis with tuberculosis; 16 per cent were classified as silico-tuberculosis; and 1 case was diagnosed as asbestosis.

The various siliceous occupations act differently in causing silicosis. The differences are due to a number of factors, such as the silica content of the dust, the size of the silica particles, the variation in the time of exposure, the presence of organic or nonsiliceous dusts mixed with silica, and the probability of the existence of an individual susceptibility.

In the group of cases studied, it was found that the most frequent complications and coexisting diseases were pulmonary tuberculosis, cardiovascular disease, emphysema, pulmonary infections, bronchiectasis, chronic pleurisy, and dental diseases.

In a study of the classification of the 97 cases of silicosis complicated by tuberculosis, it was found that in 11 per cent the tuberculous disease was classified as incipient; in 32 per cent it was moderately advanced; and in 57 per cent far advanced.

The cardiovascular system may show evidence of disease silicosis. The abnormal cardiac changes are characterized by a progressive dilatation and hypertrophy due to obstruction of the pulmonary circulation, the result of extensive fibrosis of the lungs. Of 120 living silicotics, 50 (41.7 per cent) gave evidence of disease of the heart or blood vessels; of this number 28 showed the presence of cardiac hypertrophy, dilatation, or a combination of the two cardiac conditions; 16 showed the presence of fibrosis of the myocardium; and 21 gave evidence of arteriosclerosis. Of 24 deceased silicotics on whom no post-mortem examination was done, 13 showed the presence of cardiovascular disease, of which number 6 were cases of cardiac dilatation, or a combination of hypertrophy and cardiac dilatation. In a series of 23 autopsies on silicotic subjects, reported elsewhere by the writer, hypertrophy or dilatation of the heart or a combination of the two conditions was found in 18 instances. These cardiac conditions were found in the early as well as in the advanced stages of silicosis, also in silico-tuberculosis.

While tuberculosis is the principal complicating infection in silicosis, other infections, such as pneumonia, lung abscess, and bronchiectasis, are frequently encountered. Upper and lower pulmonary tract infections were found in 56 (34 per cent) of the group of 167 silicotics;

there were 39 cases of chronic bronchitis, 11 cases of bronchiectasis, and 6 cases of chronic pharyngitis. Chronic pleurisy was present in 66 (40 per cent) of the number; this condition is ordinarily not found in uncomplicated silicosis, but in silicosis with a complicating infection.

LEUKOCYTES, The Phagocytic Activity of Human, With Special Reference to Their Type and Maturity, Hertzog, A. J. Am. J. Path. **14:** 595, 1938.

The phagocytic activity of the leucocytes from 17 cases of leucemia, 3 cases of infectious mononucleosis, 4 cases of lymphocytosis, and 2 cases of eosinophilia were investigated. Staphylococci and streptococci were used as test particles.

The mature polymorphonuclear neutrophiles showed the greatest amount of phagocytosis both as to the number of bacteria ingested per cell and as to the percentage of cells engulfing bacteria. The monocytes, eosinophiles, and metamyelocytes were also actively phagocytic.

The phagocytic activity of the myelocytes, promyelocytes, leucoblasts, and myeloblasts appears to be directly proportional to the maturity of the cell as there was a marked decrease in phagocytosis in the more immature forms. An exception was found in the histoid stem cell and histoid monoblast of leucemic reticulo-endotheliosis, as these immature cells showed an unusual degree of phagocytosis.

Phagocytosis was observed in a small per cent of mature lymphocytes. The degree of phagocytosis increased as the cell grew larger, with a corresponding increase in cytoplasm. The leucocytoid lymphocyte of infectious mononucleosis showed the great phagocytic activity. Hence, lymphocytes in their prephagocytic stage may occasionally show phagocytosis under experimental conditions.

PNEUMONIA, Recent Studies on Experimental Lobar, Robertson, O. H. J. A. M. A. **111:** 1432, 1938.

By implanting pneumococci suspended in a starch-broth paste into the terminal air sacs, it has been possible to produce in the dog a lobar pneumonia which closely resembles that which occurs in the human being.

The pneumococci are dispersed from the locus of implantation principally by the edema fluid of the early lesion, which spreads peripherally through the contiguous air passages and the pores of Cohn.

The evolution of the inflammatory lesion of the canine disease is essentially the same as that observed in human lobar pneumonia.

At the time of recovery a striking histologic change occurs in the pulmonary lesions of both man and the dog. This consists in the transformation of certain of the fixed tissue cells into free macrophages which engulf and destroy the pneumococci much more effectively than do the polymorphonuclear leucocytes. The macrophages are dependent on opsonins for their antipneumococcic activity.

The mechanism of recovery appears to be of dual nature, consisting of a generalized process, which acts to localize the infection and prevent or control bacteriemia, and a local process, the macrophage reaction, whereby the lung is enabled to rid itself of the invading microorganisms. If both processes are effective, recovery results. If either one fails, death ensues.

One attack of experimental pneumonia confers an increased resistance to subsequent infection which lasts for many months. The basis of such immunity seems to reside in the greatly accelerated macrophage reaction characterizing the recurrent lesions.

After recovery a high degree of local immunity can be demonstrated in the involved lobes; it persists only as long as the macrophages are present in the alveoli. However, the duration of the local immunity is greatly prolonged by repeated infection in the same locus. Under such circumstances a residual mobilization of macrophages is observed in the tissues of the lung.